THE EVOLUTION AND POPULATION DYNAMICS OF BACTERIAL ADDICTION COMPLEXES

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Abstract

Bacterial genomes comprise an assortment of cooperative and self-interested replicating entities (replicons). The ecology of these replicating entities most likely determines the short-term evolution of bacterial genomes. Plasmids, genetic parasites of bacteria, are ubiquitous vectors of microbial genes and are responsible for the dissemination of clinically important determinants such as antibiotic resistance. Despite their importance, there is no consistent understanding of what makes a 'successful' plasmid. Plasmids can reproduce infectiously between hosts by conjugation (horizontal transfer) and in synchrony with the host (vertical transfer). Their persistence is thought to rely upon a trade-off between horizontal and vertical transfer, where some plasmids persist as infectious but virulent elements, while others persist as immobile mutualists that resemble secondary chromosomes. As such, plasmids can have genes of varying usefulness to the host in which they may be found at any given time. This range of usefulness varies from harmful to beneficial through to cooperative traits.

A peculiar phenotype of many plasmids is post-segregational killing, where the cell is killed upon loss of the plasmid. Killing is mediated by addiction gene complexes, which comprise both a toxin and a cognate antitoxin gene. As long as both genes are expressed, cells carrying the complex remain healthy, however, loss of the complex usually entails death of the cell. Intriguingly, addiction complexes are a ubiquitous feature of bacterial replicons and are found with near uniformity across chromosomes and plasmids.

The ubiquitous presence of addiction complexes on bacterial replicons poses an intriguing evolutionary conundrum, namely, how can a trait whose principal phenotype, host-killing, be so pervasive? This thesis explores how addiction complexes affect the population dynamics of chromosomes and plasmids that carry them. We developed and analysed mathematical models of three interesting cases of addiction complex population biology: 1. the emergence of coupled toxin antitoxin gene complexes and intragenomic conflict, 2. within-host competition between

competing plasmids, 3. punishment of segregating non-cooperative alleles. Collectively, these three cases examine emergence at the level of the operon, replicon (chromosome and plasmid) and community (i.e. sociobiological traits).

The theoretical models developed herein were novel in their approach owing to their focus on the ecology of replicons (i.e. population dynamics of plasmids, chromosomes and phage). This replicon-centric approach allowed us to examine adaptation at the level of the replicon, which in turn generated novel hypotheses as to how addiction complexes may drive bacterial genome dynamics. Importantly, each model developed herein qualitatively predicted many observed behaviours that existing models do not.

Table of Contents

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CHAPTER 1

Introduction

1 BACKGROUND

The purpose of this section is to provide a focused introduction to the biology and theory of plasmids, toxin-antitoxin systems and their genetic context as part of an evolving population of bacteria. This focus will provide requisite knowledge for understanding and appreciating the body of work presented in chapters 2-5, but is not an exhaustive literature review. Each research chapter (2-4) is self-contained and will provide the background required to understand the motivation for, and importance of, the findings specific to that chapter. This section should aid the reader in understanding the research presented in the broader context of bacterial biology and the evolution of bacterial genomes.

(a) Plasmids

Over the past 60 years an extensive body of knowledge concerning the genetics and biochemistry of plasmids has been accumulated to which entire journals are dedicated (e.g. *Plasmid*). In the following sections, we will briefly cover salient details concerning plasmid biology. The intention is to introduce key concepts relevant to their population biology and provide biochemical details when needed.

It was Joshua Lederberg's formative 1952 discovery of the F-factor (Lederberg *et al.*, 1952), a plasmid of *E. coli*, which led to the advent of modern molecular genetics. F, standing for fertility, was first described as a form of bacterial sex, hence "Fertility-factor". The notion of bacterial sex arose from the prior observation that some *E. coli* strains are able to exchange genetic material in co-culture (Tatum & Lederberg, 1947), which appeared similar to what sex does in organisms that also have meiotic cycles.

Lederberg defined plasmids as "*extra-chromosomal hereditary determinants"* (Lederberg, 1952). He introduce the term "plasmid" to distinguish between nuclear and cytoplasmic inheritance (Lederberg, 1998). His seminal definition meant that any determinant inherited independent of "nuclear" genetic material was a plasmid. This included plastids, mitochondria and other genetic organelles (for an excellent historiographic review of the term "plasmid" see Lederberg, 1998).

Lederberg's definition made an important distinction for purposes of evolutionary experiments. A Lederberg-plasmid may be inherited independently of other genetic units and therefore may in part conform to different evolutionary pressures. However, Lederberg's definition is not without operational difficulties, namely, *when is a plasmid not a chromosome?* This problem may not have been foreseen at the time of Lederberg's discovery as eukaryote nuclear determinants were then thought to be neatly localised and lent themselves to a simple classification based on cellular organisation.

With the nucleic acid revolution came the molecular description of the genetic units of bacterial genomes. With this, Lederberg's definition was supplanted by physical description and plasmids became routinely described by the biochemical nature of a limited class of replicons, namely, small circular extra-chromosomal DNA. Although descriptive *anatomical* definitions of plasmids and their elements yield rich classification schema, they lack heuristic power. That is, they are unable to identify new entities at an ecological and evolutionary level of generality. For example, elements consisting of linear or single stranded DNA would suffice as plasmids using Lederberg's definition but not based on anatomical similarity to F-plasmid.

(i) Reproductive biology

Vertical transfer

Vertical transfer requires the *replication* and *segregation* of DNA to each daughter of cell division. All natural plasmids observed thus far encode determinants that ensure their faithful transmission to decedent cells in at least one host background.

Replication initiates at the "origin of vegetative replication" (*oriV*) with DNA polymerase being recruited from the cell. Plasmids encode molecules that regulate replication. In the case of plasmid ColE1, a *cis*-acting RNA replication primer precursor (activator) binds the *oriV* resulting in the recruitment of DNA polymerase, while an antagonistic *trans*-acting antisense RNA (repressor) competitively binds the primer precursor interfering with recruitment of polymerase (Tomizawa & Som,

1984). The copy-number of a plasmid, or the average number of plasmid molecules per cell, is a function of the kinetic properties of the various regulatory molecules employed. By regulating replication (i.e. copy-number), plasmids may reduce the intrinsic cost borne by the cell for maintaining the plasmid (Stewart & Levin, 1977; Van der Hoeven, 1986; Paulsson & Ehrenberg, 1998; Bergstrom *et al.*, 2000; Lili *et al.*, 2007).

Two modes of plasmid segregation exist, passive (a feature of high copy plasmids), in which plasmid copies segregate by diffusion, and active (a feature of low copy plasmids), in which plasmid copies are actively segregated by molecular machinery.

High-copy plasmids such as ColE1 maintain between 15-20 copies per cell. The stability of their inheritance is thought to rely on random assortment at cell division where plasmid molecules are binomially distributed between daughter cells (binomial segregation). Inheritance by binomial segregation is destabilized when the number of segregating units decreases. For example, the homologous recombination of plasmid molecules into multimers (multimerization) can destabilize plasmid inheritance by reducing the number of segregating units. Accordingly, some plasmids encode site-specific resolution systems that resolve multimers into monomers (Summers & Sherratt, 1984).

Low-copy plasmids such as F maintain on average 2-5 copies per cell and are unstably inherited by binomial segregation alone (Seelke *et al.*, 1982). Instead, stable inheritance is ensured by an active partitioning system. For example, the partitioning apparatus of plasmid R1, ParMC, consists of actin-like filaments that push plasmid copies to opposing polls, where, upon cell division the plasmid faithfully segregates to both daughters (Salje *et al.*, 2010).

Plasmid inheritance machinery such as copy-control and partitioning loci ensure low levels of stochastic miss-segregation of cognate plasmid replicons at cell division. Natural plasmids isolated so far have been found to transmit from parent to daughter cells with high fidelity, with empirical estimates of plasmid loss in clonal plasmid

populations are in the order of 10^{-5} per cell per generation (Turner *et al.*, 1998; Cooper & Heinemann, 2005). However, in mixed-plasmid populations these mechanisms rapidly force plasmids with cognate replication control mechanisms into distinct lineages (Condit & Levin, 1990). This phenomenon, known as *incompatibility,* appears to be a general feature of plasmids and arises as a result of reproductive self-restraint (Paulsson, 2002; Kentzoglanakis *et al.*, 2013).

It is also important to note that many plasmids encode DNA integrases or bear insertion sequences that enable them to physically integrate or recombine with other replicons (e.g. F high-frequency recombinant). In an integrated state, plasmids use the vertical inheritance mechanisms of the parent replicon, often a chromosome, and not their own.

Horizontal transfer

Plasmids are able to reproduce infectiously between cells in a process known as conjugative transfer. Conjugation entails the unidirectional replication and transfer of plasmid DNA from donor to recipient cells and is a recipient independent process (Heinemann, 1991). Conjugative plasmids are broadly categorised as transmissible, transferable and mobilizable (Clark & Warren, 1979). Transmissible plasmids can generate transconjugants through vertical replication. Transferable plasmids bear all the necessary determinants to facilitate conjugative transfer. Mobilizable plasmids, however, lack the necessary determinants to facilitate conjugal transfer, but can transfer in the presence of elements that encode them (i.e. complementation in *trans*).

All plasmids of gram-negative bacteria, transferable and mobilizable, bear an "origin of transfer" (*oriT*), a short DNA sequence at which replication initiates. The *oriT* is the only *cis*-acting determinant required for conjugative transfer. A plasmid-encode protein known as a relaxase, a phosphodiesterase, cleaves and binds at a cognate *oriT* generating a single-stranded DNA nucleoprotein complex known as a relaxosome. A coupling protein (T4CP) links the relaxosome to the cell's Type IV secretion system (T4SS) which mediates transfer of the relaxosome complex in a 5'-3' direction from donor to recipient cells (Gomis-Rüth *et al.*, 2001). DNA polymerase initiates at *oriT* bound with relaxase, whose secondary-structure is related to rolling-circle replication

initiator proteins. Upon transfer and replication, plasmid DNA is ligated by the relaxase complex completing horizontal transfer (for a detailed review of the molecular biology of conjugation see la Cruz *et al.*, 2010).

Many plasmids repress their own conjugal transfer with "fertility inhibition" systems (*fin*) that inhibit the expression of cognate *tra* genes (T4SS). Plasmid *fin*$^{-}$ mutants constitutively conjugate to the detriment of their hosts and are outcompeted by wild-type *fin*$^{+}$ plasmids in co-culture. Thus, it has been suggested that plasmids modulate their infectious reproduction in order to mitigate the cost borne by the cell (Haft *et al.*, 2009).

(ii) Plasmid persistence

Stewart & Levin's (1977) seminal study of plasmid persistence tested and modelled conjugative plasmid population dynamics in a resource-limited chemostat environment. Their study yielded an intuitive existence condition for conjugative plasmids, which in words can be described simply as; horizontal transfer has to exceed the net cost of harbouring the plasmid (reduction in vertical transfer) and loss due to miss-segregation. This simple relationship between horizontal transfer and vertical transfer is thought to be a general feature of obligate parasites (Lipsitch *et al.*, 1995). In this framework, plasmid persistence is reduced to the description of two plasmid qualities, infectivity and cost.

Estimates of plasmid transfer in unstructured environments suggest that the rate of horizontal transfer is insufficient for maintaining the cost of conjugative behaviour (Bergstrom *et al.*, 2000). As a result, it has been suggested that plasmids must bear host-adaptive traits to support their existence. This assumption has lead to what is now referred to as the *plasmid paradox*, which states that "(…) non-beneficial plasmids should be lost to purifying selection, whereas beneficial genes carried on plasmids should be integrated into the bacterial chromosome" (Harrison & Brockhurst, 2012).

However, the plasmid paradox may be an artefact of our experimental techniques and theoretical assumptions for a number of reasons. This is because, firstly, *in vitro*

estimates of conjugal transfer are thought to underestimate *in situ* rates (Sørensen *et al.*, 2005), meaning some plasmids may exist as mal-adaptive genetic parasites. Secondly, plasmids may be selected to maintain local adaptations in the presence of background selective sweeps (Bergstrom *et al.*, 2000). Thirdly, cooperative behaviours may select for horizontal transfer by increasing assortment between clone mates (Dimitriu *et al.*, 2014). With the exception of the first observation, arguments explaining plasmid-borne traits rely on either direct or indirect (e.g. cooperative/social) benefits to host fitness.

What current theory lacks, and where much more experimentation has to be done, are models that include within-host dynamics between both different plasmid replicons and plasmid interactions with other bacterial replicons (e.g. chromosomal, plasmid and phage). Of the studies conducted thus far, within-host dynamics appear to be crucial for determining plasmid success (Cooper & Heinemann, 2000; Paulsson, 2002; Cooper & Heinemann, 2005; Cooper *et al.*, 2010). Moreover, plasmids also partake directly in wider ecological conflicts. For example, male-specific bacteriophage infection, whereby plasmid-carrying cells are susceptible to infection due to the presence of plasmid-encoded conjugative pilli (Birge, 2006), suggests that there are likely to be other dimensions to plasmid fitness than host-plasmid and plasmid-plasmid alone.

(b) Toxin-antitoxin systems

Toxin-antitoxin systems can be generalised as closely linked genes that encode a stable toxin and cognate unstable antitoxin, respectively, where the toxin acts to harm the cell, while the antitoxin acts to neutralise the effect of the toxin. Cells remain viable provided expression of the antitoxin is maintained. However, insufficient expression of antitoxin, due to physiological conditions or loss of the gene, frees the cognate toxin to act resulting in cell stasis or death. Despite being ubiquitous in bacterial genomes, many toxin-antitoxin systems escape simple explanations as to their evolutionary genesis and on-going persistence (Mongold, 1992; Kussell *et al.*, 2005; Mochizuki *et al.*, 2006; Tsilibaris *et al.*, 2007; Rankin *et al.*, 2012). Since toxin activity restricts or ends a focal cell's growth, many explanations of how toxin-antitoxin systems adapt bacteria rely on social evolutionary arguments where kin-

mates benefit from cell death (Mochizuki *et al.*, 2006) or restrictive environments where arrested cell-growth is crucial for survival (Kussell *et al.*, 2005).

Toxin-antitoxin complexes are biochemically diverse and are implicated in a wide variety of phenotypes. The diversity of these phenotypes suggests it is unlikely that any single phenotype governs their evolution and population biology. Moreover, the population biology of toxin-antitoxin systems can depend on the genetic background of the bacterial population (Cooper & Heinemann, 2000; Wilbaux *et al.*, 2007) meaning their evolution could be driven by a plethora of replicon-replicon interactions for which it is difficult to examine empirically and theoretically.

(i) Biochemical mechanisms of toxin-antitoxin systems

It is likely that many biochemical mechanisms have evolved to regulate the delicate balance between toxicity and inertness of the toxin encoded in the gene pair. Hitherto, toxin-antitoxin systems have been characterised according to five biochemical mechanisms, referred to as Types I-V (for a recent review see Mruk & Kobayashi, 2013):

I. An RNA antitoxin that competitively inhibits translation of the toxin mRNA i.e. anti-sense RNA (Gerdes *et al.*, 1990);
II. A protein antitoxin that interferes with toxin activity by interaction with the toxin (reviewed in Holcík & Iyer, 1997);
III. An RNA antitoxin that interferes with toxin activity by interaction with the toxin (Fineran *et al.*, 2009);
IV. A protein antitoxin that interferes with toxin activity by disrupting the toxin's activity by binding to its target (Masuda *et al.*, 2012); and
V. An protein antitoxin with RNA-dependent endonuclease activity targeting the toxin's mRNA i.e. RNAse antitoxin (Wang *et al.*, 2012).

Although interesting in of itself, a biochemical identity based on the above type system does not necessarily correspond to an evolutionary identity. In each biochemical class of toxin-antitoxin one can find examples of different phenotypic behaviours such as post-segregational killing (PSK), stress tolerance or abortive infection, and in many cases, toxin-antitoxin systems may confer multiple phenotypes

e.g. *hok/sok* is addictive, i.e. PSK, and excludes bacteriophage T4, i.e. abortive infection (Pecota & Wood, 1996). Because of this, it is not possible to infer the evolutionary dynamics of putative toxin-antitoxin systems, which are identified based on genetic or biochemical identity.

(ii) Phenotypes of bacterial toxin-antitoxin systems

Post-segregational killing (PSK)

Post-segregational killing or bacterial addiction is where upon loss of a toxin-antitoxin gene complex, including segregational loss in daughter cells, the bacterial cell is killed by the action of the toxin (Gerdes *et al.*, 1986). When borne by plasmids, post-segregational killing may increase the prevalence of the plasmid in the population because plasmid loss is accompanied by cell death. Therefore, post-segregational killing was initially described as a plasmid stability mechanism (Gerdes *et al.*, 1986). However, unlike active partitioning mechanisms and copy-control, post-segregational killing does not increase vertical inheritance stability (Cooper & Heinemann, 2000). In mixed plasmid populations, co-infecting plasmids may rapidly segregate into different lineages due to replication control mechanisms acting on cognate origins of replication (*oriV*). Thus, when post-segregational killing is coupled to the segregation of plasmids at cell division, plasmids bearing post-segregational killing determinants will outcompete plasmids lacking a cognate antitoxin (Cooper & Heinemann, 2000; 2005; Cooper *et al.*, 2010). This model was called the "competition hypothesis" (Cooper & Heinemann, 2000).

As a phenotype, post-segregational killing may be caused by a number of different trait/environment combinations. For example, any trait that confers resistance to an environmental toxin, e.g. heavy metals or antibiotics, may act as a post-segregational killing determinant in the presence of the stressor (Harrison *et al.*, 2015). Furthermore, other traits, such as bacteriocins, are also addictive (Inglis *et al.*, 2013). Cells expressing bacteriocins toxicify their environments by exporting a toxin but remain viable provided expression of a cognate immunity gene is maintained. However, loss of the immunity function results in cell death.

Abortive infection

Abortive infection is a radically altruistic behaviour of bacteria, whereby a bacterium commits suicide upon infection by an antagonistic bacteriophage. Successful abortive infection prevents, or severely restricts, the maturation of viable phage progeny, but in doing so, renders the cell incapable of further reproduction. Abortive infection is typically mediated by toxin-antitoxin systems that respond to physiological cues associated with phage reproduction such as amino acid starvation, and operate in time periods less than needed for phage to mature (Aizenman *et al.*, 1996; Hazan & Engelberg-Kulka, 2004). The first described toxin-antitoxin system, *hok*/*sok* (Gerdes *et al.*, 1986), was later found to also confer abortive infection against T4 in *E. coli* (Pecota & Wood, 1996), and since then this dual phenotype has been observed of other abortive infection systems (Fineran *et al.*, 2009; Otsuka & Yonesaki, 2012; Dy *et al.*, 2014). As the knowledge of *in vivo* antagonistic phage-bacteria relationships is limited, it is likely that this behaviour remains unobserved for many known toxin-antitoxin systems.

Abortive infection is described as an altruistic trait as there is typically an appreciable cost associated with the constitutive expression of the necessary gene products, but this is balanced by a large benefit to the community in the form of reduced secondary infections. In support of this, a number of studies have demonstrated the importance of environmental structure and kin-selection for the evolution of abortive infection (Fukuyo *et al.*, 2012; Berngruber *et al.*, 2013; Refardt *et al.*, 2013).

Persistence

Persistence describes bacterial sub-populations that exist in a dormant or inhibited reproductive state and are phenotypically resistant to stressors that are otherwise harmful to dividing cells. The phenotypic switch between reproductive dormancy and normal cell growth is stochastic, where “persister” sub-populations persist owing to the continuous and spontaneous activation and repression of a bacteriostatic toxin (Balaban *et al.*, 2004). Persistence has been proposed as a bet-hedge against fluctuating environmental stressors (Balaban *et al.*, 2004; Kussell *et al.*, 2005), where dormant cells may survive a transient environmental stress and resume normal growth at a later time. However, persistence is highly mal-adaptive in the absence of a stressor, as dormant cells are outcompeted by cells capable of continuous

reproduction. Moreover, persistence is not suited as a strategy against prolonged stressor exposure, as persister cells eventually emerge from dormancy and become susceptible to the stressor. Thus, the evolutionary stability of persistence is thought to rely on consecutive selective sweeps for persister cells by a fluctuating environmental stressor (Kussell *et al.*, 2005). There is evidence that in some cases persister induction may be linked to the global regulator Lon (Christensen *et al.*, 2004). As such, induction of dormancy in some systems may not be random but coupled to the detection of a stressor.

Plasmid rescue

Plasmid rescue, a recently discovered phenotype of the toxin-antitoxin system *kid-kis*, is when cell division is arrested whenever the replicon bearing the toxin-antitoxin system exists at relatively too few copies to ensure faithful segregation to both daughters of cell division (Pimentel *et al.*, 2005; 2014). The resulting effect is that plasmids bearing rescue loci have increased vertical inheritance stability. The differentiating characteristic of plasmid rescue compared to persistence is that bacteriostasis is dependent on the gene dosage of the toxin-antitoxin locus and the reproductive stage of the cell.

Mechanistic details of the toxin-antitoxin *kid-kis* of plasmid R1, which is implicated in plasmid rescue, were recently elucidated by Pimentel et al. (2014). Here, the activity of the toxin Kid results in the inhibition of cell division but not protein expression in general i.e. cells fail to divide but continue to grow in size. Furthermore, Kid activity simultaneously leads to an increase in expression of the R1 replication initiating protein, RepA, meaning that both cell division is halted and replication at the plasmid's *oriV* is up-regulated.

Bacteriophage resistance

Although often considered distinct to toxin-antitoxin systems, bacterial restriction modification systems are phenotypically indistinguishable from determinants that cause post-segregational killing (Naito *et al.*, 1995). Restriction modification systems consist of a site-specific endonuclease (toxin) and cognate methylase (antitoxin). DNA encoding the recognition motif of the endonuclease is restricted (cut) unless the site has been methylated by the cognate methylase. Restriction modification systems

can act as generic phage defence determinants, where foreign phage DNA entering the cell is restricted due to the absence of methylation (Kobayashi, 2001). In cases where the methylase is overwhelmed, e.g. during phage replication, it may fail to methylate sites on the chromosome resulting in death of the cell and potentially a reduction in phage progeny i.e. abortive infection (Labrie *et al.*, 2010).

Unlike other toxin-antitoxin systems, the differential stability of toxin and antitoxin activity is thought to arise through an asymmetry between endonuclease protection and restriction. That is, the methylase must ensure that all chromosomal sites are methylated whereas the endonuclease only needs to restrict at a single site to cause death. Thus, upon loss of the gene complex dilution effects, due to cell division, increase the probability that the methylase cannot protect all recently replicated sites (Kobayashi, 2001).

Table 1. Summary of toxin-antitoxin phenotypes by toxin mode

Mode of toxicity	
Bacteriostatic	**Bactericidal**
• Stress-tolerance (persistence) • Plasmid rescue	• Post-segregational killing • Abortive infection • Phage resistance

2 WORK PRESENTED IN THIS THESIS

This thesis explores how phenotypes of toxin-antitoxin systems influence the evolution of bacterial genomes by examining their affect on the population dynamics of chromosomes and conjugative plasmids. By focusing on replicon population dynamics we were able to explore more broadly how toxin-antitoxin systems influence the evolution of bacterial genomes. Specifically, this thesis consists of three self-contained research chapters, each focusing on a different interaction that affects addiction complex population dynamics.

In Chapter 2 we develop a theoretical model to explore the evolutionary emergence of a toxin-antitoxin system. We first reproduce the results of Mochizuki *et al.* (2006) demonstrating that addiction complexes can spread on plasmids in spatially structured environments, and highlight the role of kin selection. We then consider the emergence of addiction complexes on plasmids from previously unlinked toxin and antitoxin genes. We then extend our model to allow for the movement of addiction between chromosomal and plasmid replicons and predict non-transitive "rock-paper-scissors" dynamics to be a feature of intragenomic conflict mediated by addiction complexes. This work appeared in the publication Rankin *et al.* (2012).

In Chapter 3 we go beyond single-infection dynamics and explicitly consider co-infection. Current theory suggests that addiction complexes may only evolve by kin selection in spatially structured environments, where the killing of a former host liberates resource and provides localized benefit to related individuals (Mongold, 1992; Mochizuki *et al.*, 2006). Using a mathematical model of plasmid co-infection and within-host competition we demonstrate that kin selection is unnecessary for explaining the evolution and population dynamics of addiction complexes. Instead, we find that plasmid co-infection and horizontal gene transfer permits the emergence and coexistence of distinct maladaptive plasmid replicons. In such plasmid-determined ecosystems, addictive plasmids outcompete plasmids lacking the addiction complex; moreover, addictive plasmids can even outcompete advantageous host-adaptive plasmids. We predict that within-host competition selects addiction

complexes upon reproductively competing replicons and therefore can be viewed as an essential feature of bacterial replicons.

In Chapter 4 we develop a theoretical model to explore the relationship between post-segregational killing and abortive infection, a dual phenotype of some toxin-antitoxin systems. We propose that the perfectly linked dual phenotype of many abortive infection determinants – post-segregational killing – extends the existence conditions of abortive infection by punishing non-cooperative cheats that emerge by either mutation or loss of the locus. Furthermore, we explore how plasmid-borne addiction may provide a novel mechanism to ensure the cooperative trait is maintained in the presences of plasmid defectors (i.e. plasmids that have lost the abortive infection gene) or cognate replicons that are competing for vertical reproduction.

Unless otherwise stated, references to toxin-antitoxin throughout this thesis refer to addiction complexes that cause post-segregational killing when lost.

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CHAPTER 2

The coevolution of toxin and antitoxin genes drives the emergence and dynamics of bacterial addiction complexes and intragenomic conflict

1 INTRODUCTION

Genomes comprise multiple genes which often do not share the same interest (Burt & Trivers, 2006). Such genomic conflicts are ubiquitous, and range from conflict over inheritance, such as in the case of genomic imprinting, to conflict over cell division, such as in the case of cancer where cancerous cells reproduce to the detriment of the genome as a whole.

Mobile genetic elements in bacteria provide many interesting examples of intragenomic conflict, characterized by mixed horizontal and vertical routes of transmission. Plasmids are archetypal mobile elements, and can reproduce in tandem with their host (transmitting vertically) but also independently (transmitting horizontally) often at a cost to the host (Rankin, Rocha, *et al.*, 2011). This mix of transmission routes causes a potential conflict between plasmid persistence and replication and the interests of the host chromosome (Stewart & Levin, 1977; Bergstrom *et al.*, 2000; Burt & Trivers, 2006; Lili *et al.*, 2007; Wagner, 2009; Rankin, Rocha, *et al.*, 2011).

One interesting plasmid-driven conflict is in the case of plasmid-carried toxin-antitoxin (TA) complexes (Gerdes *et al.*, 1986; 2005), where the toxin acts to harm the cell, while the antitoxin acts to neutralize the toxin. If the plasmid is lost through segregation at cell division, the stoichiometry of the toxin and antitoxin changes quickly, leading to bacteriostasis or cell death (typically due to a longer toxin half-life; Figure 1). Therefore, carriage of a TA complex does not directly enhance vertical or horizontal transmission, as its principal phenotypic effect (cell death) only occurs following the loss of the complex. As TA systems code for both an antitoxin and a toxin, it is likely that they impose a metabolic cost on the host cell, in the absence of cell death. Despite this, toxin-antitoxin systems are frequently found on plasmids (Gerdes *et al.*, 1986; 2005), raising the question of how such a system can have evolved.

A number of studies have shown or proposed that TA systems can be viewed as plasmid persistence adaptations (Kobayashi, 2001; Hayes, 2003; Magnuson, 2007;

Moritz & Hergenrother, 2007; Van Melderen *et al.*, 2009). Gerdes *et al.* (1986) demonstrated that the loss of TA cassettes induces post-segregational killing (PSK), and argued that TA cassettes therefore function as stability adaptations, 'addicting' cell lines to the TA complex. A fundamental concern with the stability/addiction hypothesis is that the PSK phenotype is only expressed following the loss of the replicon. A test of the stability hypothesis showed that TA plasmids are outcompeted by isogenic TA^- plasmids (in distinct cell lines) in the absence of conjugation (Cooper & Heinemann, 2000). However, under co-infection (within-host competition), the TA plasmid was able to outcompete and exclude the TA^- competitor from a well-mixed population, as now the PSK phenotype fell preferentially on cells carrying the TA^- plasmid (Cooper & Heinemann, 2000).

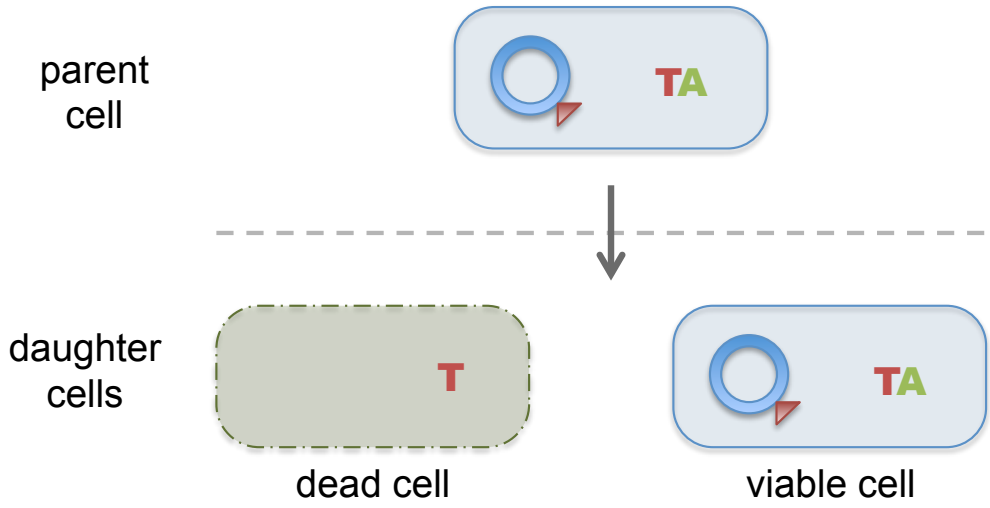

Figure 1. Schematic representation of plasmid addiction showing the presence (or absence) of the toxin (T) and antitoxin (A) within the cytoplasm. If the plasmid is lost at cell division, the antitoxin, which has a shorter half-life, quickly degrades and only the toxin is left. As a result, the daughter cell is killed.

Mongold (1992) concluded from a theoretical analysis that plasmid-level competition will not select for rare plasmid-encoded TA complexes unless they also carry host-beneficial alleles or have high rates of conjugation, and suggested that plasmid-encoded TAs are coincidental artefacts of gene transfer from chromosomes. Further theoretical analysis by Mochizuki *et al.* (2006) illustrated that the rare invasion of TA complexes could, however, be favoured by the spatial structuring of host cells. TA systems also appear frequently on the chromosomes of many bacteria (Hayes, 2003; Gerdes *et al.*, 2005; Engelberg-Kulka *et al.*, 2006; Kim *et al.*, 2009; Kolodkin-Gal *et*

al., 2009). Different reasons for why chromosomal TA systems would persist have been proposed (Hayes, 2003; Magnuson, 2007), from the role of cell death in biofilm formation (Kim *et al.*, 2009; Kolodkin-Gal *et al.*, 2009) to their role in bacterial persistence (Korch *et al.*, 2003; Keren *et al.*, 2004). Chromosomally-carried TA systems have been shown to confer host resistance to related plasmid-borne TA complexes, as the antitoxin will be present in the cytoplasm even after a TA plasmid is lost through segregation (Cooper & Heinemann, 2000; Takahashi *et al.*, 2002; Cooper & Heinemann, 2005; Saavedra De Bast *et al.*, 2008; Cooper *et al.*, 2010). TA complexes arise in a variety of different organisms, from genetic drivers in eukaryotes, to addiction complexes in bacterial plasmids, and represent a wider set of genomic conflicts (Burt & Trivers, 2006). Here we build models to describe how plasmid TA complexes evolve, and how they can be maintained in bacterial populations. We show that, given the local establishment of a plasmid-borne antitoxin gene, the full addiction complex can evolve under local competition, but that this remains unstable with respect to host resistance (i.e. chromosomal TA systems) against the toxin. If the toxin-antitoxin system is present on both chromosomes and plasmids, this ultimately leads to cycling between TA plasmids, plasmids without the TA complex, chromosomes with the TA complex and wild-type cells.

2 MODEL AND RESULTS

We build a model to examine the conditions driving the evolution and evolutionary stability of plasmid addiction. We base our model on susceptible and infected (SI) models that have been used extensively to study the evolution and persistence of plasmids and mobile genetic elements (Stewart & Levin, 1977; Bergstrom *et al.*, 2000; Lili *et al.*, 2007; Rankin *et al.*, 2010; Rankin, Rocha, *et al.*, 2011; Mc Ginty *et al.*, 2011; Svara & Rankin, 2011). We start with a population of plasmid-free hosts, with density being n_F. The density of plasmids that do not carry any toxin or antitoxin genes (which we refer to as "null", or I, plasmids) is denoted n_I and they are lost from a cell lineage (through miss-segregation at cell division) with a probability *s* and exert a cost *x* (e.g. conjugation), on their host. We assume logistic population growth, where the *per-capita* birth and death rate is given by $a-\mu N$. Here, a is the per-capita growth rate, while μ represents the density-dependent death rate and *N* the total number of cells in the population. We assume that any costs (such as the cost of

bearing a plasmid x) are small and manifest themselves as a reduced growth rate. Horizontal transfer of plasmids occurs through conjugation, at rate β. The dynamics of wild-type hosts, and hosts infected with null plasmid, are

$$\frac{dn_F}{dt} = \overbrace{n_F(a-\mu N)}^{birth\ \&\ death} - \overbrace{\beta n_I n_F}^{transfer} + \overbrace{asn_I}^{segregation}, \tag{2.1a}$$

$$\frac{dn_I}{dt} = \overbrace{n_I(a-\mu N)}^{birth\ \&\ death} + \overbrace{\beta n_I n_F}^{transfer} - \overbrace{asn_I}^{segregation} - \overbrace{xn_I}^{cost}. \tag{2.1b}$$

Full details of notation used in the model are given in Table A1 (Appendix 5(a)). Here, *N* is the total population density (i.e. $N = n_F + n_I$). In the absence of any plasmids the equilibrium density (carrying capacity) of plasmid-free cells is $n_F = a/\mu$. Plasmid-carrying cells will be able to invade this population if $\beta a/\mu > x + as$. Thus, if the rate of horizontal transfer outweighs the costs born by the plasmid (in terms of the cost of plasmid carriage *x* and the overall rate of segregation *as*), a plasmid will be able to invade. This is the general condition for plasmids to persist, which is that the rate of horizontal transfer has to exceed any net costs of harbouring a plasmid (Stewart & Levin, 1977; Lili *et al.*, 2007). More generally, the condition allows for invasion even in the absence of conjugation (i.e. when $\beta = 0$), if the plasmid carries sufficiently beneficial alleles, such that $x < 0$ and therefore $as + x < 0$. Throughout this chapter, we assume that horizontal transfer and/or the carriage of host beneficial alleles is great enough to favour plasmid persistence, and that therefore $\beta a/\mu > x + as$. This condition does not, however, take into account competition with other plasmids of the same incompatibility group in the population (later in we address this limitation by explicitly considering co-infection). So long as the invasion condition is met, the plasmid-carrying population will increase until the two populations arrive at a coexistence equilibrium with plasmid free cells persisting owing to their continuous generation from carrier cells via segregational loss.

(a) Full addiction complex

The plasmid addiction complex kills cells that lose the plasmid through segregation. We assume that cells with the complex pay an additional direct metabolic cost *c*. When the plasmid is lost through segregation (at rate *as*), we assume that the antitoxin

is quickly degraded, leaving only the toxin, and therefore the cell dies (so the loss term *as*, representing host death, is qualitatively different from the other costs *x* and *c*, which represent loss of fecundity). We assume that other cells in the population will quickly replace the dead cells resulting from PSK. In contrast, we assume that $-\mu N$ represents losses due to resource limitation and so does not permit immediate replacement.

Following other models, using assortment between strategies to model relatedness (Eshel & Cavalli-Sforza, 1982; Gardner *et al.*, 2007; Brown *et al.*, 2009; Rankin & Taborsky, 2009), we introduce the term r (where $0 \leq r \leq 1$) to denote the scale of replacement following PSK events. We use such a parameter to keep our model both tractable and general, and we assume that this replacement arises owing to the underlying spatial structure and demography (e.g. motility, life-history characteristics) of the bacteria. The most likely cause of replacement by similar cells will be if there is spatial structure, and thus our parameter r can be thought of as describing the level of assortment between strains (as such, our model has similarities to previous models incorporating explicit spatial structure; Mochizuki *et al.* (2006)). If $r = 1$, the dead cell is replaced by a cell carrying the addiction plasmid ("local" replacement, e.g. high spatial structure) where as if $r = 0$, the dead cell is replaced by a random member of the population ("global" replacement, no spatial structure) that is proportional to the frequency of the given cell type in the population (i.e. n_j/N, where j denotes the strain). To simplify our model, we further assume that cells cannot be co-infected by both null plasmids and TA plasmids. From these assumptions, the dynamics of wild-type cells, cells infected with the null plasmid, and cells infected by TA plasmid are therefore:

$$\frac{dn_F}{dt} = \overbrace{n_F(a-\mu N)}^{\substack{birth\\ and\ death}} - \overbrace{\beta n_F(n_I + n_{TA})}^{transfer} + \overbrace{asn_I}^{segregation} + \overbrace{asn_F(1-r)\frac{n_{TA}}{N}}^{\substack{global\\ replacement}}, \tag{2.2a}$$

$$\frac{dn_I}{dt} = \overbrace{n_I(a-x-\mu N)}^{\substack{birth\\ and\ death}} + \overbrace{\beta n_F n_I}^{transfer} - \overbrace{asn_I}^{segregation} + \overbrace{asn_I(1-r)\frac{n_{TA}}{N}}^{\substack{global\\ replacement}}, \tag{2.2b}$$

$$\frac{dn_{TA}}{dt} = \overbrace{n_{TA}(a - x - c - \mu N)}^{\substack{birth \\ and\ death}} + \overbrace{\beta n_F n_{TA}}^{transfer} - \overbrace{asn_{TA}}^{segregation} + \overbrace{asn_{TA}\left(r + (1-r)\frac{n_{TA}}{N}\right)}^{\substack{local\ and\ global \\ replacement}}. \quad (2.2c)$$

If the wild-type host cells and null plasmids are at the non-trivial (and positive) equilibrium, $(n_F, n_I, n_{TA}) = (n_F{}^*, n_I{}^*, 0)$, the addiction complex will be able to invade if

$$r > \frac{c}{as}. \quad (2.3)$$

As long as this condition holds, the addiction plasmid will both outcompete, and be immune to invasion from, null plasmids (Figure 2). The simple rule, described in inequality (2.3), shows that local replacement is a requirement for the complex to evolve, and high segregation rates, and low costs of addiction, will favour the evolution of the addiction complex. A critical criterion in assessing inequality (2.3) is the magnitude of segregational loss, as. In the absence of co-infection, as is due to the rare failure of the segregational machinery during cell division, with estimates of as being at least as low as 10^{-3}/hr (Condit & Levin, 1990), rendering inequality (2.3) irrelevant for all but the most costless plasmids. In contrast, the rate of segregational loss in co-infected cells is far higher, as the normal functioning of segregational machinery will lead to the rapid separation of incompatible plasmids into distinct lineages, with *s* tending to 0.5/hr for doubly infected cells (Condit & Levin, 1990; Mongold, 1992), greatly favouring the likelihood of TA invasion. Later in the paper we explicitly introduce co-infection dynamics.

It is interesting to note the similarity between inequality (2.3) and Hamilton's rule $R > C/B$ (Gardner *et al.*, 2011). In this case, C is the cost of expressing an altruistic behaviour (in this case, the overall cost c of maintaining the addiction complex), R is the relatedness (represented by r) between an actor (the addiction-carrying plasmid, which kills its host after being segregated) and a recipient (a cell which replaces the dead cell) and the benefit of the behaviour B of expressing the addiction complex (in this case as, the rate of segregation loss of plasmids in the population, dependent in turn on co-infection).

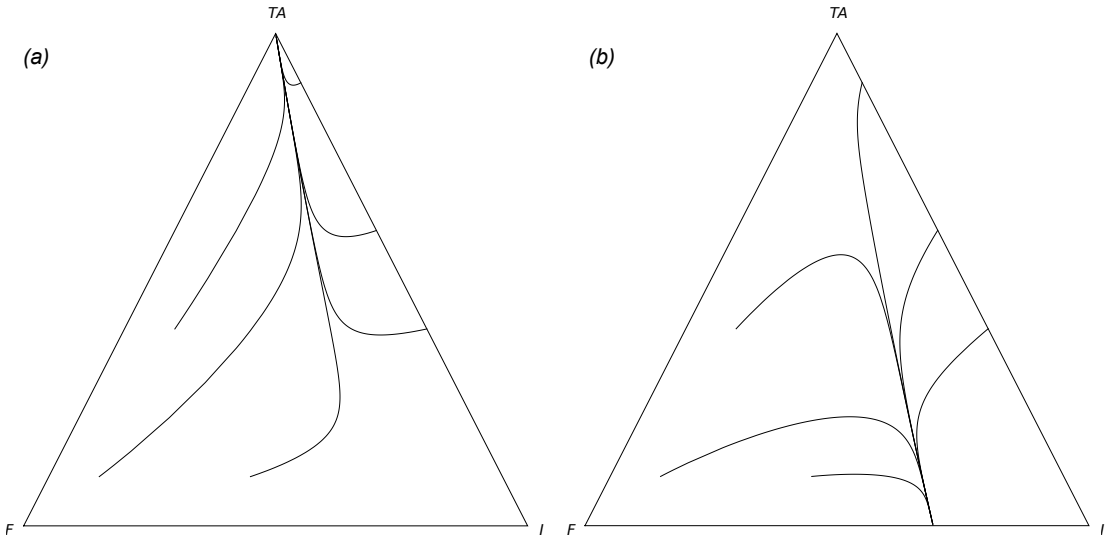

Figure 2. Numerical simulations drawn as phase diagrams in triangular *Inset* showing proportions of *F*, *I* and *TA* for (a) dominance by a TA encoding plasmid (where $r = 0.75$ and $ras > c$) and (b) dominance by a null (non-TA) plasmid (where $r = 0.1$ and $ars < c$). Initial conditions: (*I*(0), *TA*(0)) = (0.1, 0.1), (0.1, 0.4), (0.1, 0.9), (0.4, 0.1), (0.4, 0.6), (0.6, 0.4). Remaining parameters are: *a*=1/hr, *a/μ*= carrying capacity = 10^8/ml, β=7.5*10^{-12}/bacterium/hour/ml, *c*=10^{-4}/hr, *x*=10^{-4}/hr, *s*=3*10^{-4}/hr.

(b) Separating the complex

The preceding analysis dealt with the TA complex as being a single entity. However, the evolution of TA systems represents a "chicken-egg" paradox: without the antitoxin, the toxin cannot evolve, but the antitoxin has no use in a context lacking the toxin. We therefore now assume that the complex is made up of two separable genes, one coding for the toxin (denoted by subscript *T*), and the other coding for the antitoxin (denoted by subscript *A*). What are the possible evolutionary trajectories of these two genes? To establish a TA complex, we argue that either the T or A gene alone must offer a direct advantage in some but not all of the environments encountered by an evolving plasmid population, thus breaking the deadlock between two individually costly genes. We formalize our argument using the 'antitoxin first' scenario in the following text. In the discussion, we highlight a parallel 'proto-toxin first' solution to this 'chicken-egg' paradox.

To build the 'antitoxin-first' argument, we begin by assuming that the toxin gene is lethal when alone (we relax this assumption in the discussion). We now assume that

n_A cells are viable, with the plasmid-carried A gene imposing a direct cost y and a potential benefit z. The dynamics of the antitoxin and the TA complex can then be described by the equations:

$$\frac{dn_A}{dt} = \overbrace{n_A(a+z-x-y-\mu N)}^{\substack{birth\\ and\ death}} + \overbrace{\beta n_F n_A}^{transfer} - \overbrace{asn_A}^{segregation} + \overbrace{asn_A(1-r)\frac{n_{TA}}{N}}^{\substack{global\\ replacement}} \quad (2.4a)$$

$$\frac{dn_{TA}}{dt} = \overbrace{n_{TA}(a+z-x-c-\mu N)}^{\substack{birth\\ and\ death}} + \overbrace{\beta n_F n_{TA}}^{transfer} - \overbrace{asn_{TA}}^{segregation} + \overbrace{asn_{TA}\left(r+(1-r)\frac{n_{TA}}{N}\right)}^{\substack{local\ and\ global\\ replacement}} \quad (2.4b)$$

If $z > y$ there is a net benefit to the plasmid carrying the antitoxin gene (we separate y and z in order to explicitly distinguish the costs and benefits of the antitoxin gene). An antitoxin allele could generate resistance or stabilizing benefits z to the host-plasmid lineage for a variety of reasons, for example by conferring antibiotic or bacteriocin resistance to the cell.

Modifying equations (2.2)*a,b*, the dynamics of the wild-type cells and null plasmid are then:

$$\frac{dn_F}{dt} = \overbrace{n_F(a-\mu N)}^{\substack{birth\\ and\ death}} - \overbrace{\beta n_F(n_I+n_A+n_{TA})}^{transfer} + \overbrace{as(n_I+n_A)}^{segregation} + \overbrace{asn_F(1-r)\frac{n_{TA}}{N}}^{\substack{global\\ replacement}} \quad (2.5a)$$

$$\frac{dn_I}{dt} = \overbrace{n_I(a-x-\mu N)}^{\substack{birth\\ and\ death}} + \overbrace{\beta n_F n_I}^{transfer} - \overbrace{asn_I}^{segregation} + \overbrace{asn_I(1-r)\frac{n_{TA}}{N}}^{\substack{global\\ replacement}} \quad (2.5b)$$

If both wild-type cells, and those infected with the null plasmids, are at the non-trivial (and positive) equilibrium (i.e. $n_F{}^*$ and $n_I{}^*$), we can see that the antitoxin will invade subpopulations simply if $z > y$. Thus, the antitoxin must generate some direct benefit in order to outcompete the null plasmid. Given a homogenous population of host cells (where z is constant across all host cells), and $z > y$, the A plasmid will replace null plasmids across all subpopulations, and the resistance or antitoxin gene will be uniformly present across all plasmids. Assuming that the TA plasmids also get a

benefit z from carrying the A gene on the plasmid (as part of the TA complex), the full TA complex can still invade (when n_F^* and n_A^* are at equilibrium) if $ras > c - y$.

In contrast, if the cellular environments are heterogeneous (and z varies across cells in different environments), the A plasmid can invade subpopulations where A yields a benefit ($z > y$, Figure 3c-f) and fail in others (in particular, where A does not yield a benefit and $z = 0$, and is outcompeted by null plasmids Figure 3a,b). The resulting heterogenous distribution of the A allele provides a context for the emergence and spread of the TA complex. Specifically, the full complex can emerge if plasmids in subpopulations supporting the A plasmid then go on to acquire the toxin gene (Figure 3c-f), and can then expand in subpopulations where $z = 0$ (and thus $n_A = 0$), whenever $ras > c$ (e.g. Figure 3b). The cellular heterogeneity results in subpopulations that are locally adapted to some local stressor (e.g., carriage of the A gene in Figure 3e,f). The toxin gene, in combination with the antitoxin, can then invade (e.g. Figure 3b). The importance of environmental heterogeneity in breaking "chicken-egg" obstacles to toxin-resistance trait evolution has also been proposed in the context of immuno-manipulative pathogens (Brown *et al.*, 2008). In this immunological context, host heterogeneity allows the specialization of pathogen strains on distinct immunological challenges (generating differential resistance or "antitoxin" settings). Strains specialized on the most challenging environments can then invade more benign environments by triggering the environmental stressors to which they are already adapted (via immuno-manipulation or, in the present study, toxin production). Toxin-antitoxin loci may evolve in an analogous manner. A diverse array of plasmids are known to code for specific resistance phenotypes to heterologously distributed environmental stresses (Martínez, 2008; 2009; Rankin, Rocha, *et al.*, 2011). In consequence, these resistance determinants (i.e., A plasmids) are locally adaptive in a restricted and challenging subset of environments. Plasmids that encode resistance determinants in response to a locally present toxin may then co-opt expression of the toxin, permitting the TA encoding plasmid to invade benign environments lacking resistance whenever $ras > c$.

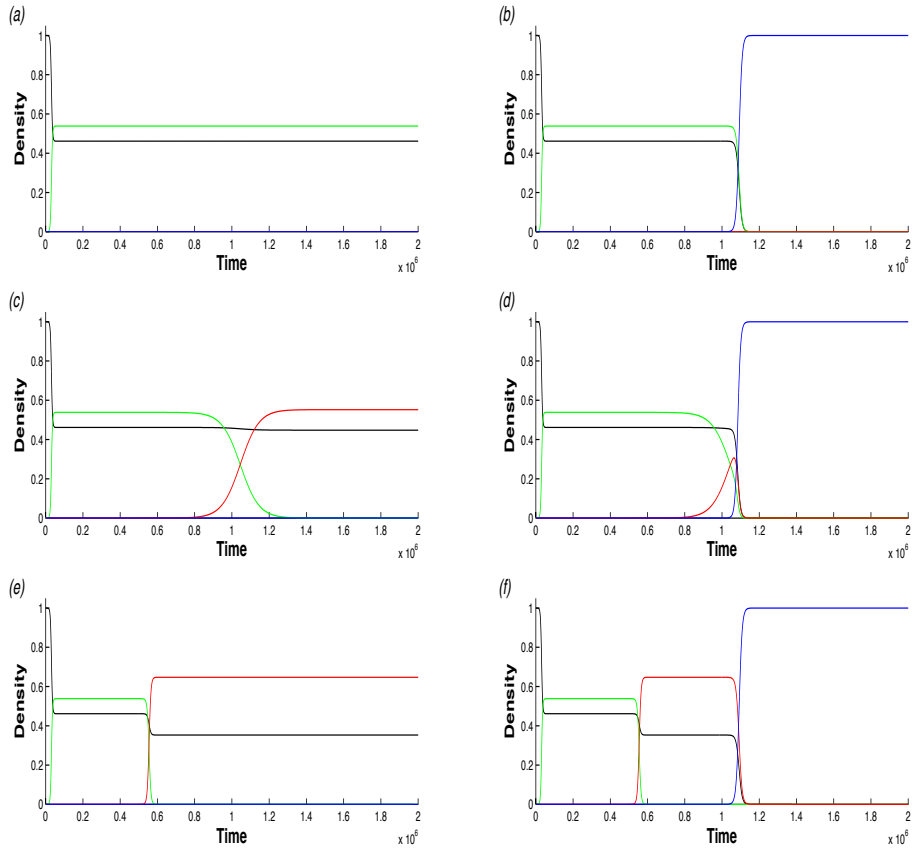

Figure 3. Invasion dynamics of plasmids carrying antitoxin genes (*A*) and addiction complexes (*AT*) as a function of antitoxin direct benefit *z* and relatedness *r*. (a,b), no direct benefit of antitoxin gene (*z* =0). (c,d), intermediate benefit (*z* = 10^{-5}). (e,f), high benefit (*z* =2*10^{-4}). (a,c,e), low relatedness (*r* = 0.25). (b,d,f), high relatedness (*r* = 0.75). The population starts off with wild-type cells (*F*) and null plasmids (*I*) at equilibrium. After this strains are added from rare: $n_A(t=5*10^5)=10^{-5}$, $n_{AT}(t=10^6)= 10^{-5}$. Other parameters as in Figure 2, plus *y*=0. The line colours and type refer to: solid-black: *F* wild-type cells, solid-green: *I* null plasmids, solid-red: *A* antitoxin plasmids, solid-blue: *AT* plasmids carrying the addiction complex. Densities are scaled to the carrying capacity of uninfected cells (*a/μ*).

(c) Host resistance to the toxin

Following the invasion and dominance of a host population by a TA plasmid, we now ask whether a host resistance trait (i.e. a chromosomally coded antitoxin) can evolve in benign environments lacking the exogenous stressor (i.e. $z = 0$; if the direct benefits of the A gene are sufficiently large, then there is little puzzle surrounding its acquisition, even in the absence of circulating TA plasmids). One simple mechanistic route to the establishment of a chromosomal antitoxin gene is via transposition from plasmid to the chromosome of A and/or TA. TA systems are frequently observed to be carried on bacterial chromosomes (e.g. Gerdes *et al.*, 2005). Host bacteria carrying the TA system chromosomally can potentially resist the lethal effects of TA plasmid loss, as the cell retains the ability to produce the antitoxin (Cooper & Heinemann, 2000; Takahashi *et al.*, 2002; Cooper & Heinemann, 2005; Saavedra De Bast *et al.*, 2008; Cooper *et al.*, 2010).

To begin our analysis of host resistance, we consider the case where the TA plasmid has gone to fixation (see equation (2.2)c), at an equilibrium density $n_{TA}{}^{*} = (a - c - x)/\mu$. We now consider a rare population of resistant uninfected hosts n_R, which upon infection with the TA plasmid will generate a lineage of infected resistant hosts n_{RTA}. Note that "resistance" implies only that the host lineage can survive the loss of the T encoding plasmid, and does not imply any resistance to infection. The density of individuals with resistance on the chromosome is given by:

$$\frac{dn_R}{dt} = \overbrace{n_R(a - y_C - \mu N)}^{\substack{birth \\ and\ death}} - \overbrace{\beta n_R(n_{TA} + n_{RTA})}^{transfer} + \overbrace{asn_{RTA}}^{segregation} + \overbrace{asn_R(1 - r)\frac{n_{TA}}{N}}^{\substack{global \\ replacement}} \tag{2.6a}$$

$$\frac{dn_{RTA}}{dt} = \overbrace{n_{RTA}(a - x - y_C - c - \mu N)}^{\substack{birth \\ and\ death}} + \overbrace{\beta n_R(n_{TA} + n_{RTA})}^{transfer} - \overbrace{asn_{RTA}}^{segregation} + \overbrace{asn_{RTA}(1 - r)\frac{n_{TA}}{N}}^{\substack{global \\ replacement}} \tag{2.6b}$$

Here, y_C is the cost of carrying the resistance determinant on the chromosome. We assume that the cost of the antitoxin alone is less than the direct costs of the full TA. We can look at the invasion criteria by considering the Jacobian matrix of the

combined system comprising of equations (2.2)c and (2.6). The resistance gene will invade if the dominant eigenvalue of this matrix is positive, evaluated when n_{TA} is at equilibrium, $n_{TA}{}^{*} = (a-c-x)/\mu$, and $n_R \to 0$ and $n_{RTA} \to 0$. This is the case if:

$$as(1-r) \geq y_C \qquad (2.7)$$

Inequality (2.7) suggests that lower spatial structure (i.e. lower r) and higher segregational loss (higher as) favours the evolution of host resistance. Interestingly, if $as-y_C > asr > c$, we predict cycling among strains. Specifically, a plasmid carrying the TA complex will be able to invade a population of null plasmids, after which any cell that develops resistance to the TA plasmid will also be able to invade. Once resistance establishes itself in a population, a null plasmid (or a plasmid only with the antitoxin gene) will be able to outcompete a TA plasmid as null plasmids do not pay the cost c of expressing the TA complex (as long as $c > 0$). Once null plasmids have invaded, wild-type cells will be able to invade, outcompeting cells with TA on the chromosome (as they do not pay the cost of the complex on the chromosome, y_C).

(d) Numerical simulation of full dynamics

The interactions between strains n_F, n_{TA} and n_R display a non-transitive form of competitive advantage labeled "rock-paper-scissors" dynamics after the popular children's game (Kerr *et al.*, 2002). In our case n_{TA} beats n_F and n_I, n_R beats n_{TA}, n_F beats n_R, etc. These non-transitive interactions are closely akin to three-strain models of bacteriocin production in bacteria, where killer (toxin-antitoxin), resistant (antitoxin only) and sensitive (neither) can cycle and potentially co-exist in spatially structured populations (Kerr *et al.*, 2002; Czárán *et al.*, 2002; Czárán & Hoekstra, 2003; Kirkup & Riley, 2004). This highlights the non-transitivity of toxin-antitoxin systems in general, and strain cycling will be common regardless of whether the TA complex is involved in the production of bacteriocins or in PSK. To examine whether TA systems could lead to cell cycling, we built (using MATLAB) a numerical model with the dynamics described above.

The results of the initial invasion of the addiction complex can be seen in Figure 3b. This shows that a population of plasmid-free wild-type cells is first invaded by a null

plasmid, and then is invaded by the TA complex, which then dominates the population and excludes all other cells. Figure 4 shows the dynamics of our full model, where the antitoxin can be carried on the chromosome, for both low relatedness (Figure 4a, $r = 0.25$) and high relatedness (Figure 4b, $r = 0.75$). In the case of low relatedness, wild-type cells and cells carrying the antitoxin plasmid prevail, and cannot be invaded by other types (Figure 4a), while when relatedness is high (and inequality (2.3) is fulfilled), the TA plasmid can invade (as in Figure 4b).

However, in this case, once the addiction plasmid has invaded, a cell with the antitoxin encoded on the chromosome can invade. The resulting population also harbours the addiction plasmid, but at a lower density, and in combination with plasmid-free cells carrying the resistance gene. After this, a plasmid with the antitoxin gene invades, which facilitates the invasion of plasmid free wild-type cells, coexisting with plasmids carrying the antitoxin gene. Once this state of coexistence is reached the addiction complex can once more invade. Both our analytical models, and the numerical simulation in Figure 4b, demonstrate that plasmid addiction will involve cycling between the different genes and that, over longer evolutionary time-scales, addiction complexes are inherently unstable. The numerical results presented in Figure 3 and Figure 4 introduce rare strains at regular intervals (every 10^4 generations) in order to demonstrate that cycling is possible between strains. To test the robustness of cycling to random introduction of new strains, we modified our model to start with all strains present at a low density (i.e. with a density of 10^{-4}). Interestingly, null plasmids never invade in this model, as they are always outcompeted by A plasmids. Figure 5 shows that addiction complexes, when carried on the chromosome and on a plasmid, can give rise to strain cycling.

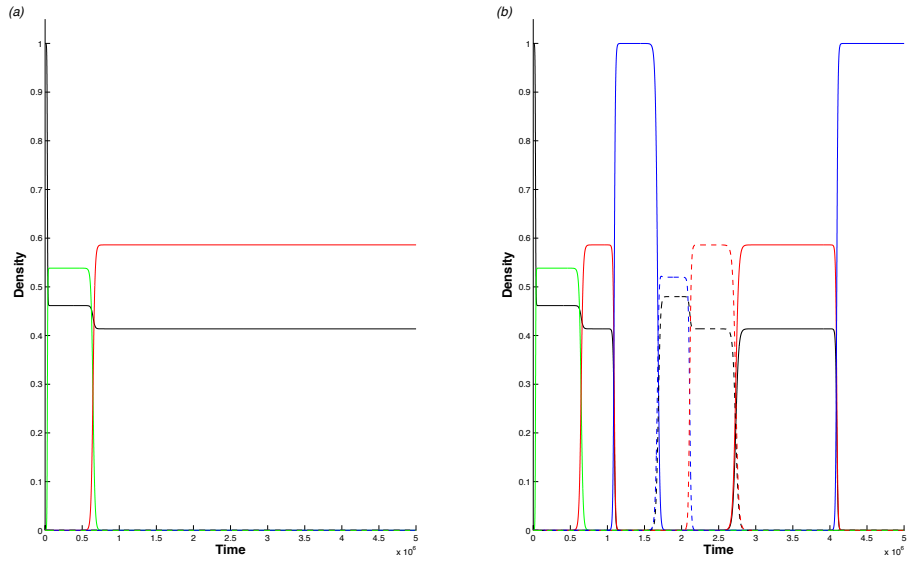

Figure 4. Numerical simulation of the full model (see Appendix 5(b) for equations and method) illustrating cycling between strains for (a) the case where addition cannot invade ("low" relatedness, r=0.25) and (b) for the case where plasmid addiction can invade ("high" relatedness, r=0.75), and drives strain cycling. The population starts off with wild-type cells (*F*) and null plasmids (*I*) at equilibrium. After this strains are added from rare: $n_A(t=5\text{x}10^5)= 10^{-5}$, $n_{AT}(t=10^6)=10^{-5}$, $n_R(t=1.5\text{x}10^6)= 10^{-5}$, $n_{RA}(t=2\text{x}10^6)= 10^{-5}$, $n_F(t= 2.5*10^6)=10^{-5}$, $n_I(t= 3*10^6)= 10^{-5}$ $n_{AT}(t= 3.5*10^6)= 10^{-5}$ and $n_{AT}(t= 4*10^6)= 10^{-5}$. Other parameters as for Figure 2, plus $z = 7.5\times10^{-5}$, y=5*10^{-5}, y_C=0. The line colours and type refer to: solid-black: *F* wild-type cells, solid-green: *I* null plasmids, solid-red: *A* antitoxin plasmids, solid-blue: *TA* plasmids carrying the addiction complex, dotted-black: *R* plasmid-free cells with resistance on the chromosome, dotted-green: *RI* cells with resistance on the chromosome carrying null-plasmid, dotted-red: *RA* cells with resistance on the chromosome carrying an antitoxin plasmid, dotted-blue: *RTA* cells with resistance on the chromosome carrying the addiction plasmid.

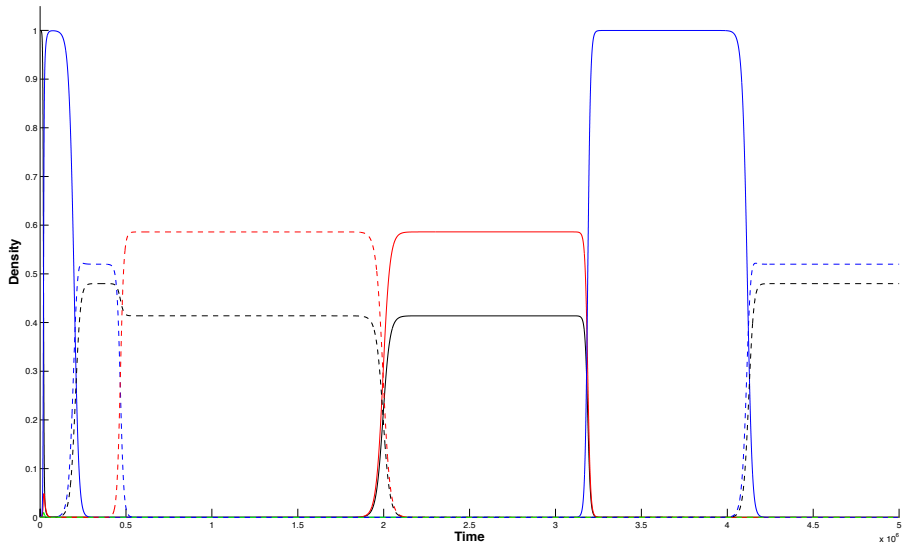

Figure 5. Numerical simulation of the full model illustrating non-transitive dynamics (see Appendix 5(b) for equations and method). Wild-type cells start at equilibrium density (n_F=1) and all other cells begin at a low density in the model (i.e. 10^{-4}). Solid grey lines represent plasmid-free cells with resistance on the chromosome while dotted grey lines represent cells infected with plasmids carrying the addiction complex. Parameters as for Figure 4. The line colours and type refer to: solid-black: *F* wild-type cells, solid-green: *I* null plasmids, solid-red: *A* antitoxin plasmids, solid-blue: *TA* plasmids carrying the addiction complex, dotted-black: *R* plasmid-free cells with resistance on the chromosome, dotted-green: *RI* cells with resistance on the chromosome carrying null-plasmid, dotted-red: *RA* cells with resistance on the chromosome carrying an antitoxin plasmid, dotted-blue: *RTA* cells with resistance on the chromosome carrying the addiction plasmid.

(e) Co-infection and segregational partitioning

We earlier demonstrated that the ability of a TA plasmid to invade a resident null plasmid population would depend critically on the rate of segregational loss (inequality (2.3)), and we commented that this rate will in turn depend on the prevalence of co-infection. While our inequality (2.3) can be interpreted generally (with s varying with the extent of co-infection), we now explicitly explore the synergistic interaction between within-host competition and spatial structuring.

Here, we assume equal partitioning, where cells infected with two different plasmids give rise to one daughter cell with one plasmid and one with the other. As above, we assume that if the TA plasmid is lost from a cell, as is the case when the daughter cell only inherits the null plasmid from a cell containing both the TA and the null plasmid, then that daughter cell will be killed. We further retain the previous assumption that, if a cell is killed by PSK, another viable neighbouring cell (I or TA) will replace the dead cell based on the genetic structure r of TA-carrying cells. In addition, we assume no miss-segregation and no wildtype strains (i.e., $s = n_F = 0$. As all bacteria now carry plasmids, we simplify the notation by describing the maximal growth rate as $\alpha = a - x$). Together, these assumptions yield the following dynamical equations

$$\frac{dn_I}{dt} = \overbrace{n_I(\alpha - \mu N)}^{\substack{birth \\ and\ death}} - \overbrace{\beta n_I\left(\frac{n_{ITA}}{2} + n_{TA}\right)}^{co-infection} + \overbrace{n_{ITA}(\alpha - c)(1-r)\frac{n_I}{n_I + n_{TA}}}^{\substack{global \\ replacement}} \tag{2.8a}$$

$$\frac{dn_{TA}}{dt} = \overbrace{n_{TA}(\alpha - c - \mu N)}^{\substack{birth \\ and\ death}} - \overbrace{\beta n_{TA}\left(\frac{n_{ITA}}{2} + n_I\right)}^{co-infection} + \overbrace{n_{ITA}(\alpha - c)}^{segregation} + \overbrace{n_{ITA}(\alpha - c)\left(r + (1-r)\frac{n_{TA}}{n_I + n_{TA}}\right)}^{\substack{local\ and\ global \\ replacement}} \tag{2.8b}$$

$$\frac{dn_{ITA}}{dt} = \overbrace{n_{ITA}(-\alpha - c - \mu N)}^{\substack{birth \\ and\ death}} + \overbrace{\frac{\beta}{2}n_{ITA}(n_I + n_{TA}) + 2\beta n_I n_{TA}}^{co-infection} \tag{2.8c}$$

In order to evaluate whether the TA system will be able to invade from rare, we take the Jacobian matrix of equations (2.8) evaluated at ($n_I = \alpha/\mu,\ n_{TA} = n_{ITA} = 0$). If

the dominant eigenvalue of this matrix is positive, then a rare TA system will be able to invade, which is the case when the following inequality holds:

$$r > \frac{2(c\mu)^2 - (\alpha\beta)^2 + \alpha c\mu(4\mu + 5\beta)}{4\alpha\mu\beta(\alpha - c)} \quad (2.9)$$

In qualitative agreement with our earlier inequality (2.3), we see that the TA complex can spread if relatedness is sufficiently high (inequality (2.9)). More generally, a little algebra shows that this critical value r^* is monotonically decreasing with β, and when β equals zero, the TA complex cannot invade for any cost. This result implies that increasing transmission favours the kin-selected advantage to an invading TA lineage, due to the increasing incidence of co-infected cells (higher s, in the terms of inequality (2.3)).[1]

3 DISCUSSION

Intrageneomic conflict can be found in all genomes (Burt & Trivers, 2006). Our model examines the evolution of toxin and antitoxin systems in plasmids, and the role they may play in intragenomic conflict. Our results demonstrate that a key way that these costs can be mitigated is if, once a cell dies as a result of the complex, it is more likely to be replaced by a cell carrying the same complex. This is also true of the model incorporating partitioning between incompatible plasmids: relatedness is an important component of whether TA systems can evolve. TA plasmids can invade if there is a sufficiently high chance, r, that the dead cell will be replaced by a cell carrying the TA complex. If a cell is killed by the TA complex and replaced at random from the entire population, then the cost of bearing the TA complex cannot be outweighed by the gain brought by killing cells not carrying the plasmid. Genetic structure of the plasmid population (generated both by structuring of the bacterial population and by co-infection of neighbouring bacteria) is therefore an important component of plasmid competition and the evolution of TA complexes. The r in our model is equivalent to relatedness (and can be seen as a measure of assortment between strains, or genetic similarity between the cell killed by PSK and the strain

[1] Interestingly, inequality (2.9) also suggests TA plasmids may invade when relatedness is absent (i.e. $r = 0$). Invasion in the absence of relatedness is examined in detail in the following chapter, Chapter 3.

which replaces it; Gardner *et al.* (2007), Ranking & Taborsky (2009)). In the case of plasmids, not only does spatial structure increase associations between local cells, but the act of transmitting plasmids can also increase such genetic structure (Nogueira *et al.*, 2009; Rankin, Ginty, *et al.*, 2011).

Understanding how toxin-antitoxin complexes arise has been a perplexing issue so long as the antitoxin serves no benefit in the absence of the toxin, and the toxin only acts to harm the cell (and the plasmid itself). No matter the benefits of the combination of toxin and antitoxin, if each trait alone is costly, then the stepwise evolution of the combined complex remains problematic. We argue that this apparent barrier to a stepwise evolution of TA complexes can be overcome if one of the traits offers a direct advantage in some but not all the environments encountered by an evolving plasmid population (Brown *et al.*, 2008). In the results section we outline an 'antitoxin-first' scenario, were the A gene serves some environmental resistance function that is beneficial in some stressful subset of host environments (Figure 3c,d). Consistent with this scenario, plasmids often confer resistance to patchily distributed environmental stressors (Martínez, 2008; 2009). From this widespread plasmid/A (resistance) association we conjecture that plasmid/TA associations are an elaboration involving the recruitment of a T gene to extend the local advantage of the partner A gene into new, less extrinsically stressful environments, that cannot support the antitoxin trait alone (Figure 3b).

Thus far we have assumed the toxin is harmful in all contexts. Now, we relax this assumption in order to consider a 'toxin-first' route to TA complex evolution. The apparent toxicity of certain genes can be context specific, where the gene product may confer a lethal, deleterious or even beneficial phenotype depending on the environment. For example, conditionally expressed proteins that transport nitrogenous compounds when preferred sources of nitrogen are limiting can be both adaptive and either deleterious or lethal depending on the environment. In a nitrogen limiting environment in which amino acids may be prevalent, an amino acid transporter is adaptive. However, in the same environment with toxic amino acid analogues, expression of the transporter may be deleterious or lethal (e.g. Heinemann *et al.*, 1994). Similarly, transporters can suppress the effects of mutations in biosynthetic

pathways, being adaptive when the essential metabolite cannot be made, but deleterious when a toxic analogue is also present (e.g. Lemeignan *et al.*, 1993). In both cases toxicity is an environment-dependent side-effect of an otherwise adaptive trait. Similarly to the former case of an initially adaptive antitoxin, environmental heterogeneity (in this case heterogeneity in toxicity) permits the gene that can have a toxic effect to exist in a subset of environments without the need for a coupled antitoxin. Therefore, in the absence of an antitoxin the toxin gene is constrained to environments where it is benign. However, when the toxin and antitoxin genes are linked on a plasmid, the plasmid is able to invade populations of cells that lack resistance to the toxin whenever $ras > c$. The 'toxin first' scenario is of potentially greater importance for the evolution of type 1 TA systems where the antitoxin (antisense RNA) presumably has no function besides suppressing toxin expression. In both evolutionary scenarios, environmental heterogeneity (of either T or A effect) is necessary to explain the evolutionary origins of TA systems on plasmids. Once TA plasmids evolve, they can potentially have beneficial effects on bacterial host populations. For example, plasmid-carried TA complexes have been found to be associated with microbial public good production (secreted proteins), and can be viewed as a mechanism to enforce costly cooperative behaviours (Smith, 2001; Nogueira *et al.*, 2009; Mc Ginty *et al.*, 2011; Platt *et al.*, 2012).

Following the spread of a functional TA complex in a host population due to sufficient local replacement of killed cells (i.e. $ras > c$), the establishment of an antitoxin gene on the host chromosome can be favoured, which protects cells in the event of segregational loss of the TA plasmid (Cooper & Heinemann, 2000; Takahashi *et al.*, 2002; Cooper & Heinemann, 2005; Saavedra De Bast *et al.*, 2008; Cooper *et al.*, 2010). If this antitoxin gene is otherwise costly to the host cell, selection on this gene is likely to be reversed as soon as the TA plasmid is sent sufficiently into decline, in turn favouring the subsequent invasion by the susceptible wild-type and thus opening the potential for "rock-paper-scisssors" cycling coexistence of sensitive, killer and resistant strain-types (Kerr *et al.*, 2002; Kirkup & Riley, 2004). This can help to explain the widespread occurrence of TA genes on bacterial chromosomes (Gerdes *et al.*, 2005; Magnuson, 2007): if a TA complex jumps from the plasmid to a chromosome, that host will have the potential to reduce

the effects of the toxin, and thus avoid PSK. While many alternative explanations have been given for the occurrence of chromosomal TA systems (e.g. Magnuson, 2007), our model is the first to examine their role as a co-evolutionary mechanism to avoid PSK from TA plasmids and is in accordance with empirical studies that show chromosomally encoded TA loci prevent within-host competition (Cooper *et al.*, 2010). These results are also eminently testable via simple competition experiments, as the predicted non-transitive competitive hierarchies translate directly into invasion experiments: Rare n_{TA} can invade resident n_F and resident n_I; rare n_R can invade resident n_{TA}; rare n_F can invade resident n_R.

From the perspective of a given plasmid, segregational loss and cell death are equivalent (since both result in an equivalent loss of direct fitness to the plasmid). As our model shows, plasmids gain an indirect benefit from harming their host when other isogenic plasmid-carrying cells benefit: while PSK has immediately negative consequences for its ex-host, it confers an indirect benefit on other isogenic plasmids by increasing their local frequency. It is worth noting the similarity between TA systems on plasmids and cytoplasmic incompatibility (CI). For example, in the bacteria-induced CI found in many insects, bacteria such as *Wolbachia* are only transmitted through the female line, i.e. through eggs rather than sperm (Werren, 1997). This has led to adaptations in bacteria to kill males produced by a female, in order to favour their spread to the next generation. It is widely acknowledged that spatial structure plays an important role in the evolution of CI (Turelli & Hoffmann, 1991; Frank, 1996; Reuter *et al.*, 2008; Engelstädter & Telschow, 2009). Bacteria that induce CI don't benefit directly from killing males, as they are not transmitted through the male line. In a similar way, TA plasmids do not gain an advantage from killing cells that no longer carry a plasmid. In both plasmid addiction and CI, it is necessary that related individuals (either CI-carrying females or TA-carrying cells, respectively) benefit from PSK.

Our model goes beyond the scope of other models by examining both the origin and persistence of the full TA complex from its antitoxin and toxin-components, and exploring the notion that the widely observed prevalence of TA systems on bacterial chromosomes (Gerdes *et al.*, 2005) could be a host adaptation to plasmid addiction

complexes. Co-evolutionary arms races are common in many systems (Dawkins & Krebs, 1979; Arnqvist & Rowe, 2002; Bergstrom & Lachmann, 2003; Archetti & Brown, 2004), and it is likely that hosts will try to resist the costs of PSK inflicted by TA complexes.

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5 APPENDIX

(a) Parameters and variables used

Symbol	Description
n_F	Density of wild-type cells (F)
n_I	Density of cells infected with the null plasmid (I)
n_A	Density of cells infected with a plasmid bearing the antitoxin gene only (A)
n_{TA}	Density of cells infected with a plasmid bearing the addiction-complex (TA)
n_R	Density of cells with the antitoxin on the chromosome (R)
n_{RI}	Density of resistant cells (R) infected with null plasmid (I)
n_{RA}	Density of resistant cells (R) infected with a plasmid bearing the antitoxin gene only (A)
n_{RTA}	Density of resistant cells (R) infected with a plasmid bearing the addiction complex (TA)
a	Per capita population growth rate
μ	*Per capita* density-dependent death rate
N	Total density of cells in the population (in the full system it is $N = n_F + n_I + n_A + n_{TA} + n_R + n_{RI} + n_{RA} + n_{RTA}$).
β	Rate of horizontal gene transfer
s	Segregation rate
r	Scale of replacement of killed cell
x	Cost of bearing a plasmid
z	Benefit of antitoxin, carried on a plasmid
c	Cost of plasmid addiction-complex
y	Cost of antitoxin
y_C	Cost of antitoxin when carried on the host chromosome

Table A1 List of notation used in the model

(b) Full system of ordinary differential equations

$$\frac{dn_F}{dt} = \overbrace{n_R(a-\mu N)}^{\substack{birth\\ and\ death}} - \overbrace{\beta n_F(n_{TA}+n_{RTA}+n_A+n_{RA}+n_I+n_{RI})}^{transfer} + \overbrace{as(n_I+n_A)}^{segregation} + \overbrace{asn_F(1-r)\frac{n_{TA}}{N}}^{\substack{global\\ replacement}} \tag{5.1a}$$

$$\frac{dn_I}{dt} = \overbrace{n_I(a-x-\mu N)}^{\substack{birth\\ and\ death}} + \overbrace{\beta n_F(n_I+n_{RI})}^{transfer} - \overbrace{asn_I}^{segregation} + \overbrace{asn_I(1-r)\frac{n_{TA}}{N}}^{\substack{global\\ replacement}} \tag{5.1b}$$

$$\frac{dn_A}{dt} = \overbrace{n_A(a-x+z-\mu N)}^{\substack{birth\\ and\ death}} + \overbrace{\beta n_F(n_A+n_{RA})}^{transfer} - \overbrace{asn_A}^{segregation} + \overbrace{asn_A(1-r)\frac{n_{TA}}{N}}^{\substack{global\\ replacement}} \tag{5.1c}$$

$$\frac{dn_{TA}}{dt} = \overbrace{n_{TA}(a-x+z-c-\mu N)}^{\substack{birth\\ and\ death}} + \overbrace{\beta n_F(n_{TA}+n_{RTA})}^{transfer} - \overbrace{asn_{TA}}^{segregation} + \overbrace{asrn_{TA}}^{local\ replacement} + \overbrace{asn_{TA}(1-r)\frac{n_{TA}}{N}}^{global\ replacement} \tag{5.1d}$$

$$\frac{dn_R}{dt} = \overbrace{n_R(a-y_C-\mu N)}^{\substack{birth\\ and\ death}} - \overbrace{\beta n_R(n_{TA}+n_{RTA}+n_A+n_{RA}+n_I+n_{RI})}^{transfer} + \overbrace{as(n_{RI}+n_{RA}+n_{RTA})}^{segregation} + \overbrace{asn_R(1-r)\frac{n_{TA}}{N}}^{\substack{global\\ replacement}} \tag{5.1e}$$

$$\frac{dn_{RI}}{dt} = \overbrace{n_{RI}(a-x-y_C-\mu N)}^{\substack{birth\\ and\ death}} + \overbrace{\beta n_R(n_I+n_{RI})}^{transfer} - \overbrace{asn_{RI}}^{segregation} + \overbrace{asn_{RI}(1-r)\frac{n_{TA}}{N}}^{\substack{global\\ replacement}} \tag{5.1f}$$

$$\frac{dn_{RA}}{dt} = \overbrace{n_{RA}(a-x+z-y_C-\mu N)}^{\substack{birth\\ and\ death}} + \overbrace{\beta n_R(n_A+n_{RA})}^{transfer} - \overbrace{asn_{RA}}^{segregation} + \overbrace{asn_{RA}(1-r)\frac{n_{TA}}{N}}^{\substack{global\\ replacement}} \tag{5.1g}$$

$$\frac{dn_{RTA}}{dt} = \overbrace{n_{RTA}(a-x+z-c-y_C-\mu N)}^{\substack{birth\\ and\ death}} + \overbrace{\beta n_R(n_{TA}+n_{RTA})}^{transfer} - \overbrace{asn_{RTA}}^{segregation} + \overbrace{asn_{RTA}(1-r)\frac{n_{TA}}{N}}^{\substack{global\\ replacement}} \tag{5.1h}$$

System (5.1) was numerically integrated using the recurrence relation $n_i(t+1) = n_i(t) + \delta \frac{\partial n_i(t)}{\partial t}$, where δ is small and i denotes the particular strain (see Figure 4 and Figure 5 for examples of integration). The total density of bacteria within the full system is $N = n_F + n_I + n_A + n_{TA} + n_R + n_{RI} + n_{RA} + n_{RTA}$. Equations (5.1)*a-d* correspond to equations (2.5)*a,b* and (2.4)*a,b* in the main text for densities n_F, n_I, n_A and n_{TA} respectively. These equations were extended to include horizontal transfer of plasmids from resistant bacteria. Equations (5.1)*e,h* correspond to equations (2.6)*a,b* in the main text for densities n_R and n_{RTA} respectively. To complete the ecological dynamics of the full model two additional genotypes are introduced: n_{RI}, the density of resistant cells bearing null plasmid, and, n_{RA} the density of resistant cells bearing an antitoxin-only plasmid. The dynamics of n_{RI} and n_{RA} cells are analogous to n_I and n_A except resistant cell lines bear the addition cost, y_c, of expressing the complete toxin-antitoxin system. Again, these equations were extended to include horizontal transfer from non-resistant cell lines.

CHAPTER 3

Co-infection and horizontal transfer can maintain bacterial addiction complexes

1 INTRODUCTION

Bacterial genomes abound with an assortment of self-interested infectious elements whose reproductive success is not necessarily aligned with that of the cell (Rankin *et al.*, 2011). Conjugative plasmids, infectious genetic elements of bacteria, reproduce in synchrony with the host (vertical transfer) and infectiously by conjugation (horizontal transfer). Plasmid fitness is thought to rely on a trade-off between cost of plasmid carriage, which is an increasing but saturating function of the rate in which it transmits to new hosts (Stewart & Levin, 1977; Lili *et al.*, 2007). The trade-off hypothesis predicts that plasmids will accumulate genes that either benefit cell reproduction or increase infectivity i.e. genes that increase vertical and horizontal transfer (Turner *et al.*, 1998).

Co-infection is where two or more parasites infect a single host and is known to drastically alter the selective pressures that drive parasite evolution (Read, 2001; Brown *et al.*, 2002; Mideo, 2009; Alizon *et al.*, 2013). As each host contains a finite exploitable resource, parasites may evolve strategies to optimise their transmission in the presence of competitors. Competition between co-infecting parasites leads to asymmetries in parasite reproduction within co-infected hosts. This asymmetry is driven by determinants that either affect the mortality or fecundity of competing parasites (interference competition) or more efficiently exploit host resources (over-reproduction). If co-infection is common, traits that enhance transmission within co-infected hosts may be paramount for deciding parasite success (Alizon *et al.*, 2013).

An important factor of plasmid success is the ability to transmit, both vertically and horizontally, in the presence of diverse range of genetic elements (Cooper & Heinemann, 2000; Dionisio *et al.*, 2002; Cooper & Heinemann, 2005; Cooper *et al.*, 2010). Conjugative plasmids of all types readily co-infect their bacterial hosts, however, not all plasmids are able to coexist in a single lineage. Related plasmids possess a form of collective reproductive self-restraint that limits their intra-cellular concentration. Intracellular concentrations of plasmids are maintained at levels that prevent over-exploitation of cell resources but ensures faithful inheritance (Paulsson, 2002). In mixed plasmid infections, with cognate origins of replication, the intra-

cellular concentration of each plasmid genotype is maintained on average at half the copy number of the origin. Thus, co-infecting plasmids that share cognate origins of replication are in reproductive conflict. One mechanism that is known to effect the outcome of this conflict is post-segregational killing (Cooper & Heinemann, 2000).

Post-segregational killing is where upon loss of a toxin-antitoxin gene complex the cell is killed by the action of the toxin. When toxin-antitoxin complexes are carried by any two replicons in conflict, then the cell or a daughter of cell division may be killed. Applied to plasmids, post-segregational killing is directed against incompatible replicons that have co-infected the cell, resulting in the death of daughters that only inherit the plasmid with no antitoxin gene. In experimental studies, plasmids bearing post-segregational killing determinants have been shown to outcompete plasmids lacking immunity to the action of the toxin (Cooper & Heinemann, 2000).

Mathematical models of plasmid population dynamics typically model plasmid transmission using a single genetic background, that of a plasmid in an otherwise plasmid-less host (Stewart & Levin, 1977; Van der Hoeven, 1986; Bergstrom *et al.*, 2000; Lili *et al.*, 2007). Despite the importance of within-host competition in determining plasmid success, there are very few models that include direct interactions between plasmid genotypes.

In this chapter, we go beyond single-infection dynamics (as discussed in Chapter 2) and explicitly consider co-infection dynamics. Using a mathematical model of plasmid co-infection, we describe the dynamics of toxin-antitoxin mediated competition in bacteria. Our model demonstrates 4 key qualitative behaviours that current models fail to predict: 1. We show that plasmid borne toxin-antitoxin systems can invade even if mal-adaptive to the cell and in the absence of kin-selection (in contrast to Mochizuki *et al.*, 2006; Rankin *et al.*, 2012); 2. Plasmid incompatibility and post-segregational killing mediates within-host interference competition between plasmids; 3. Once established, toxin-antitoxin bearing plasmids are resistant to invasion by plasmids lacking immunity to the action of the toxin i.e. hysteresis (also in contrast to Mochizuki *et al.*, 2006; Rankin *et al.*, 2012); 4. Addictive alleles are able to invade and outcompete host beneficial alleles when borne by plasmids.

Moreover, our model predicts three distinct outcomes upon invasion of a toxin-antitoxin bearing plasmid, depending on the force of infection (the rate of horizontal transfer). Firstly, for modest rates of transfer, singularly infected hosts and co-infected hosts of each plasmid genotype may coexist. Secondly, for intermediary rates of transfer, TA plasmids purge null plasmids. Finally, for high rates of transfer TA plasmids dominate but null plasmids may persist owing to infectious reproduction only.

In summary, our model, for the first time, analytically describes the dynamics of within-host competition between plasmids and shows that addiction complexes radically alter plasmid fitness. These findings corroborate with the hypothesis that toxin-antitoxin systems accumulate upon conjugative plasmids as a plasmid-level adaptation driven by within-host competition (Cooper & Heinemann, 2000). This is in contrast to single infection models which narrowly predict plasmids to accumulate genes that adapt the cell or increase infectivity. Our model suggests that co-infection is an integral part of the evolutionary and population dynamics of plasmids.

2 MODEL & RESULTS

We build a model to examine the conditions driving the evolution and evolutionary stability of plasmid-borne addiction complexes. We base our initial model on 'susceptible and infected' (SI) models that have been used extensively to study the evolution and persistence of plasmids and mobile genetic elements (Stewart & Levin, 1977; Bergstrom *et al.*, 2000; Lili *et al.*, 2007). We then extend this basic model to include co-infection and within-host competition between cognate plasmids.

(a) Plasmid dynamics in the absence of co-infection

We start with a population of plasmid-free hosts, with density being n_F. The density of hosts that bear a self-transmissible plasmid (which we refer to as 'null', or I, plasmid) is denoted n_I. We assume logistic population growth, where the per capita birth and death rate is given by $a - \mu N$. Here, a is the *per capita* growth rate, and μ is a density-dependent death rate; both a and μ are constrained to be positive. The total cell density of the population is N, where $N = n_F + n_I$. Plasmids exert a cost x on their host (e.g. conjugation); we assume this cost manifests as a reduction in fecundity. Plasmids are assumed to be lost from a cell lineage through segregational loss at cell division with a probability s. Horizontal transfer of plasmids occurs through conjugation following mass-action kinetics at rate β. With these assumptions, the dynamics of wild-type hosts, n_F, and hosts infected with null plasmid, n_I, are

$$\frac{dn_F}{dt} = \overbrace{n_F(a-\mu N)}^{birth\ \&\ death} - \overbrace{\beta n_I n_F}^{transfer} + \overbrace{asn_I}^{segregation}, \tag{2.1a}$$

$$\frac{dn_I}{dt} = \overbrace{n_I(a-\mu N)}^{birth\ \&\ death} + \overbrace{\beta n_I n_F}^{transfer} - \overbrace{asn_I}^{segregation} - \overbrace{xn_I}^{cost}. \tag{2.1b}$$

The stability properties of a system of linear ordinary differential equations (ODEs) can be assessed by linearization around a point of interest. Let $s(\mathbf{M})$ denote the spectral bound of a matrix $\mathbf{M}$ (i.e. the maximum real part of all eigenvalues), where the spectral bound of the Jacobian matrix of a system of ODEs evaluated at point x, $s(\mathbf{J}_x)$, indicates the stability of point x. If $s(\mathbf{J}_x) < 0$, then the point is stable, whereas if $s(\mathbf{J}) > 0$ the point is unstable (for an example of this technique see Hurford *et al.*, 2010).

Model (2.1) has three positive steady states. First, the trivial equilibrium, $\mathcal{E}_0 = \left(n_{F_0}^* = 0, n_{F_0}^* = 0\right)$, with $s(\mathbf{J_0}) = a$, is unstable given positive growth, $a > 0$. Second, the exterior equilibrium, $\mathcal{E}_1 = \left(n_{F_1}^* = a/\mu, n_{I_1}^* = 0\right)$ represents a monoculture of plasmid-free hosts, with $s(\mathbf{J_1}) = \beta\frac{a}{\mu} - x - as$. Third, the interior coexistence equilibrium $\mathcal{E}_2 = \left(n_{F_2}^*,\ n_{I_2}^*\right)$[2] represents a bi-culture of plasmid-bearing and plasmid-free hosts. The stability of $\mathcal{E}_1$ is lost when $s(\mathbf{J_1}) > 0$, which coincides with $\mathcal{E}_2$ gaining stability in a transcritical bifurcation which is described by

$$\beta\frac{a}{\mu} > as + x, \tag{2.2}$$

thus, if per capita horizontal transmission at equilibrium, $\beta n_{F_1}^*$, is greater than the cost borne by plasmid carriage and segregational loss, $x + as$, a plasmid will be able to invade. Inequality (2.2) is similar to that derived by previous studies (Stewart & Levin, 1977; Lili *et al.*, 2007; Chapter 2) and is the general condition for plasmid persistence. In the case of mal-adaptive plasmids, $x > 0$, horizontal transfer must outweigh segregational loss and plasmid cost, in the case of adaptive plasmids, $x < 0$, vertical transmission alone may be sufficient to negate the effect of segregational loss i.e. $as + x < 0$. So long as the invasion condition is met, the plasmid-carrying population will increase until the two populations arrive at equilibrium $\mathcal{E}_2$, the interior coexistence equilibrium, with plasmid-free cells persisting owing to their continuous generation from carrier cells via segregational loss (Figure 6).

[2] An analytical expression for E_2 was computed using Mathematica, however, it has been omitted due to its size.

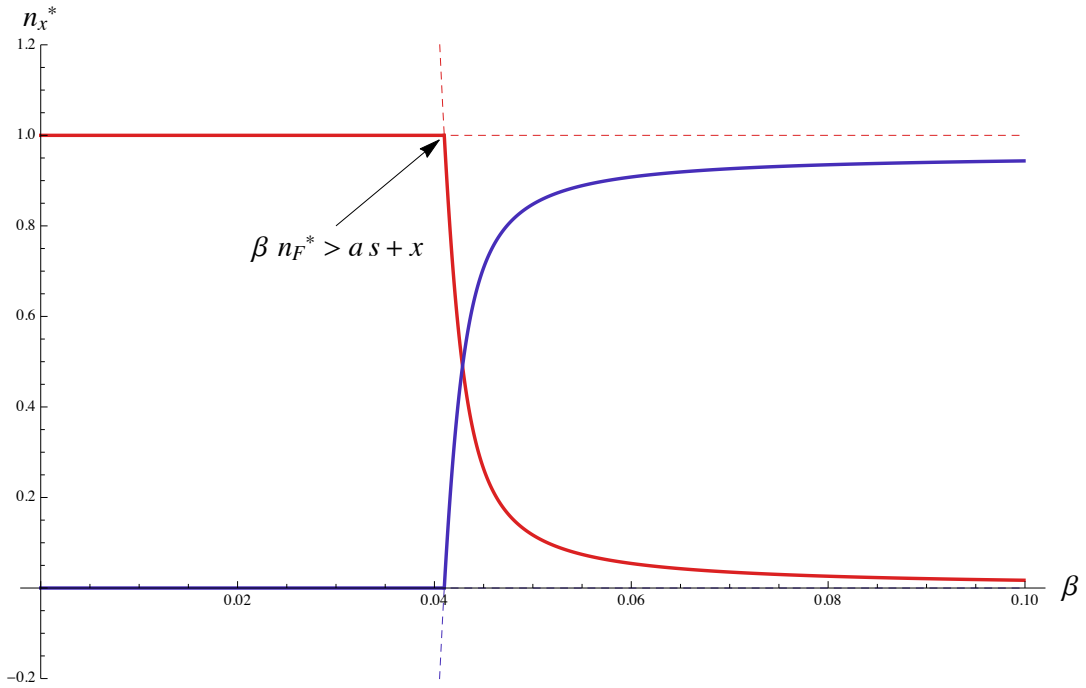

Figure 6 Continuation of equlibria $\mathcal{E}_1$ and $\mathcal{E}_2$ by horizontal transfer, β (i.e. bifurcation diagram of model (2.1)). Plasmids may invade plasmid-free cells at equilibrium, n_F^*, whenever $\beta n_F^* > as + x$. Upon invasion, plasmid-free and plasmid bearing hosts coexist where plasmid-free cells persist due to their continuous generation from carrier cells via segregational loss. Densities of plasmid-free and plasmid bearing hosts, n_F^* and n_I^*, in red and blue respectively. Parameters $a = 1, x = 0.04, s = 10^{-4}$ and $\mu = 1$.

(ii) Invasion of a toxin-antitoxin encoding plasmid

We now consider the evolutionary fate of a TA-bearing plasmid that spontaneously arises at the coexistence equilibrium $\mathcal{E}_2$. Except for the TA locus, TA plasmids and null plasmids are isogenic (i.e. genetically identical), where the production of the TA complex incurs a metabolic cost, c, which we assume manifests as a reduction in the host's fecundity. We assume that host killing upon segregational loss of TA plasmids (post-segregational killing) is absolute and instantaneous and that plasmids are unable to co-infect already infected cells (we relax this assumption in the following section). With these assumptions we introduce a third equation to model (2.1) describing the rate of change of TA plasmids with density n_{TA} and a total cell density of $N = n_F + n_I + n_{TA}$,

$$\frac{dn_{TA}}{dt} = \overbrace{n_{TA}(a-\mu N)}^{birth\ \&\ death} + \overbrace{\beta n_{TA} n_F}^{transfer} - \overbrace{asn_{TA}}^{segregation} - \overbrace{(x+c)n_{TA}}^{cost}. \qquad (2.3)$$

In the absence of co-infection, a mutant TA plasmid that spontaneously arises at the coexistence equilibrium $\mathcal{E}_2$ may invade when (for a derivation see Appendix 5(a))

$$c < 0, \qquad (2.4)$$

that is, unless the focal mutation increases vertical stability or horizontal transfer (i.e. a decrease in segregation, s, or an increase in conjugal transfer, β) more costly plasmid mutants are unable to invade. This is in agreement with results derived previously by Van der Hoeven (1984).

Conjugation is driven by donors and does not require an active participation by recipients (Heinemann & Ankenbauer, 1993). The necessary and sufficient molecular machinery for plasmid transfer is expressed by donor cells. However, recipient cells may bear determinants that restrict conjugal transfer, such as surface or entry exclusion, which typically reduce transfer frequencies by 10 – 1000 fold (Heinemann & Ankenbauer, 1993). Single-infection models implicitly assume surface or entry exclusion is absolute. This assumption introduces frequency-dependent selection against secondary infecting plasmids. When a plasmid invades a naïve population of cells (the plasmid-free equilibrium $\mathcal{E}_1$), all cells are competent recipients. Upon plasmid invasion, naïve cells and plasmid-bearing cells converge to the positive equilibrium $\mathcal{E}_2$, where the proportion of naïve and plasmid-bearing cells vary owing to plasmid cost, stability and infectivity. Since the first plasmid exists at the positive equilibrium $\mathcal{E}_2$, the number of recipient cells, i.e. naïve cells, is strictly less than the number present in a naïve-only population, meaning that the second plasmid is at a distinct disadvantage in terms of potential recipient cells i.e. $n_{F_1}^* > n_{F_2}^*$. In essence, simplifying assumptions inherent in single-infection models prevent them from adequately describing the effects of within-host dynamics.

Equation (2.3) is similar to equation (2.2*c*) developed in Chapter 2 with the exception that we assume no genetic structure and therefore no indirect forms of selection, such as kin-selection, acting upon the invading mutant. Hitherto, genetic structure has been suggested to be a necessary feature of evolutionary models of post-segregational killing (Mongold, 1992; Mochizuki *et al.*, 2006; Cooper *et al.*, 2010; Rankin *et al.*, 2012; Chapter 2). These models assume that limited dispersal of clone mates and horizontal gene transfer generates a positive assortment among individuals carrying the TA gene complex, and thus, when hosts are killed by post segregational-killing,

their resources are preferentially utilised by cells bearing the TA gene complex. The evolution of TA gene complexes as "selfish alleles", where the TA locus benefits from the resources freed by the apoptosis of former hosts, has been criticised for having "serious quantitative difficulties" as rates for gene loss, by mutation, recombination or segregation are predicted to generate a near immaterial benefit (Magnuson, 2007). These results are in part due to mutant plasmids not being able to exploit plasmid-bearing hosts as conditions (2.2) and (2.4) do not take into account co-infection with, and competition between, other plasmids of the same incompatibility group in the population. We now address this limitation by explicitly considering co-infection in a two-plasmid system.

(b) Plasmid dynamics assuming co-infection and within-host competition

Plasmids readily co-infect their bacterial hosts (Heinemann & Ankenbauer, 1993), however, not all plasmids are able to coexist in a single lineage. Cognate plasmids frequently segregate into distinct lineages. This *incompatibility* of inheritance is due to plasmid replication control mechanisms acting on cognate origins of replication (*oriV*). Incompatibility arises from binomial assortment of plasmid copies at cell division. The strength of incompatibility (i.e. the number of generations required for two distinct lineages to emerge) is dependent on the copy-number, where low-copy plasmids segregate rapidly (1-3 generations) while medium to high copy plasmids may coexist for many (>50) generations (Velappan *et al.*, 2007)[3]. Plasmid incompatibility can be viewed as a form of intragenomic conflict brought about by collective reproductive restraint (Kentzoglanakis *et al.*, 2013). When two distinct incompatible plasmids co-infect a host, their replication is co-repressed in *trans*, thereby limiting the number of copies of each plasmid genotype to the copy number of the shared replication origin. For isogenic replication origins, reduction in vertical fecundity is symmetric with each plasmid being equally susceptible to repression. However, determinants that alter the symmetry of this conflict, such as addiction complexes, can dramatically alter the success of their encoding plasmid (T. Naito *et al.*, 1995; Y. Naito *et al.*, 1998; Cooper & Heinemann, 2000; 2005; Cooper *et al.*, 2010). We now explore the population dynamics and evolutionary stability of a

[3] In this case two plasmids sharing a pBR322 origin, which maintains plasmids between 15-70 copies per cell, were able to persist within a single lineage for 50 generations.

plasmid borne addiction complex while accounting for co-infection and within-host competition.

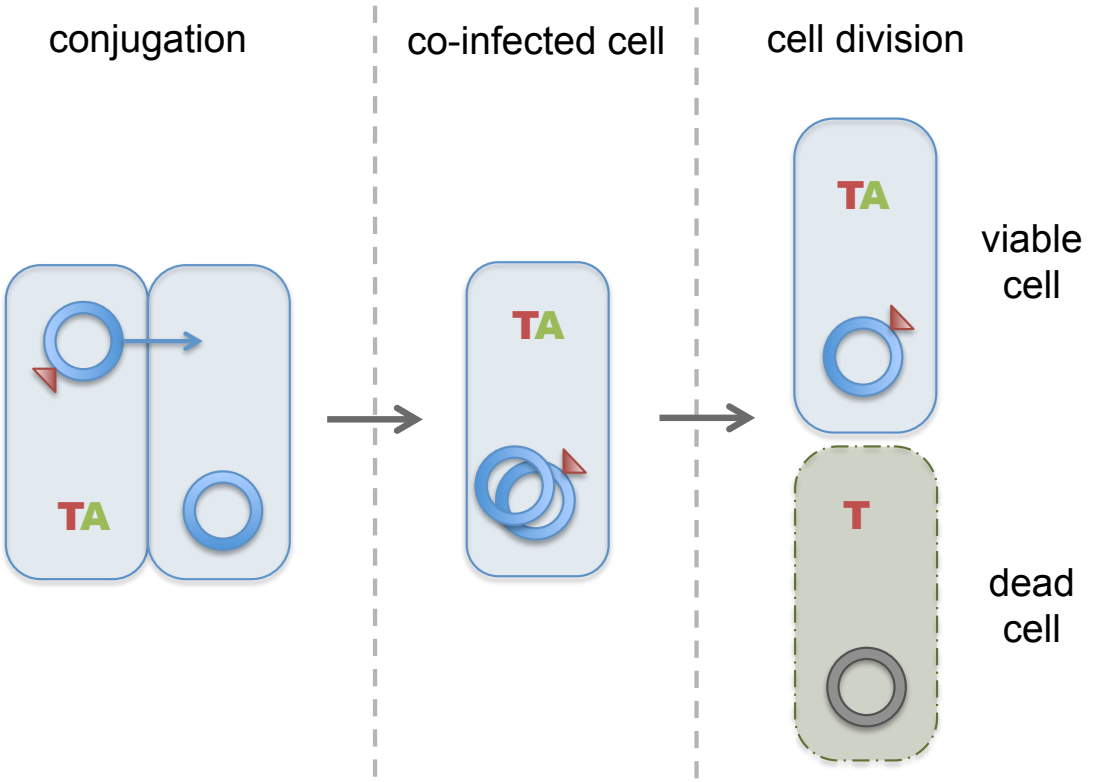

Figure 7. Plasmid competition driven by addiction complexes. (Left) A plasmid encoding a TA complex (red triangle) transfers via conjugation to a host with null plasmid and expresses the toxin (red 'T') and antitoxin (green "A"). (Middle) The resulting exconjugant is a co-infected host bearing both null and TA plasmids. (Right) Upon cell division the plasmids segregate to distinct daughter cells (transconjugants) due to replication control and partitioning mechanisms. As null plasmid lacks immunity to the longer-lived toxin, the toxin is freed to act resulting in cell death.

We now introduce an addictive isogenic toxin-antitoxin encoding plasmid (TA plasmid) with density of hosts bearing only the TA plasmid being n_{TA}. The addiction complex exerts a cost, c, which manifests as a reduction in the maximal growth rate of the host. TA plasmids readily infect hosts carrying null plasmid and vice versa resulting in co-infected hosts, with density being n_{ITA}. Plasmids of co-infected cells are equally partitioned, where cell division gives rise to one daughter cell with only a null plasmid and one daughter cell with only a TA plasmid. We assume that daughters of co-infected cells that fail to inherit a TA plasmid are killed immediately by post-segregational killing (Figure 7). We further assume that horizontal transfer of each plasmid is inversely proportional to the number of plasmids, e.g. each plasmid

transfers at half the rate of transfer that it would in a singularly infected host, $\frac{1}{2}\beta$. Finally, we introduce a simplifying assumption, namely, that the density of plasmid-free hosts at the interior coexistence equilibrium of model (2.1) is negligible ($n_{F_2}^* \cong 0, n_{I_2}^* > 0$). In general, vertical inheritance of natural plasmids in clonal populations is extremely stable with estimates of segregational loss, s, being less than 10^{-4} per cell per generation under conditions of exponential growth (Condit & Levin, 1990; Turner *et al.*, 1998). The interior equilibrium density of plasmid-free hosts, $n_{F_2}^*$, diminishes as s decreases, where the limit of equilibrium densities of plasmid-free and plasmid-carrying hosts, $n_{F_2}^*$and $n_{I_2}^*$, as s approaches 0 is $\lim_{s\to 0} n_{F_2}^* = 0$ and $\lim_{s\to 0} n_{I_2}^* = \frac{a-x}{\mu}$ respectively (i.e. the density of plasmid-free hosts at the interior coexistence equilibrium of model (2.1), $n_{F_2}^*$, tends to zero as the rate of segregation, s, becomes small). In contrast, the rate of segregational loss in co-infected cells is far higher as the normal functioning of replication machinery (copy-number control and partitioning loci) leads to the rapid separation of incompatible plasmids into distinct lineages, with s being as high as 0.5 per cell per generation for doubly infected cells (Condit & Levin, 1990). Therefore, segregation is now modeled implicitly as the cell division of co-infected hosts. As all bacteria now carry plasmids, we simplify the notation describing maximal growth rate as $\alpha = a + x$. Together, these assumptions yield the following dynamical equations (now with the total density of hosts being $N = n_I + n_{TA} + n_{ITA}$)

$$\frac{dn_I}{dt} = \overbrace{n_I(\alpha - \mu N)}^{birth\ \&\ death} - \overbrace{\beta n_I\left(n_{TA} + \frac{1}{2}n_{ITA}\right)}^{co-infection}, \tag{2.5a}$$

$$\frac{dn_{TA}}{dt} = \overbrace{n_{TA}\big((\alpha - c) - \mu N\big)}^{birth\ \&\ death} - \overbrace{\beta n_{TA}\left(n_I + \frac{1}{2}n_{ITA}\right)}^{co-infection} + \overbrace{(\alpha - c)n_{ITA}}^{segregation}, \tag{2.5b}$$

$$\frac{dn_{ITA}}{dt} = \overbrace{n_{ITA}(-(\alpha - c) - \mu N)}^{segregation\ \&\ death} + \overbrace{\frac{1}{2}\beta n_{ITA}(n_I + n_{TA}) + 2\beta n_I n_{TA}}^{co-infection}. \tag{2.5c}$$

Model (2.5) describes how the densities of null plasmid bearing hosts, n_I, TA plasmid bearing hosts, n_{TA}, and co-infected hosts, n_{ITA}, change in time. We now examine the

evolutionary stability and population dynamics of these two distinct co-infecting plasmids.

(i) Numerical analysis

Some intuition of model (2.5) can be gained by first exploring a subset of the parameter space using numerical integration. Consider the following scenario. A TA-encoding plasmid, which confers a cost representative of empirically derived estimates, $c = 0.01$ given $\alpha = 1$ (Cooper & Heinemann, 2000), enters a population of cells uniformly infected by a null plasmid. Time series show that the TA plasmid may invade given sufficient horizontal transfer, and that upon invasion, hosts singularly infected by null plasmid are eliminated from the population, $n_I{}^* = 0$, while hosts infected by the TA plasmid converge to one of two equilibria depending on the rate of horizontal transfer, β, with respect to its critical threshold, β^* (i.e. the minimum rate of horizontal transfer sufficient for invasion). If the rate of horizontal transfer is modestly greater than its critical threshold, $\beta > \beta^*$, TA plasmids persist solely within singularly infected hosts, $n_{TA}{}^* > 0$ and $n_{ITA}{}^* = 0$ (Figure 8A). Whereas, if the rate of horizontal transfer is much greater than its critical threshold, $\beta \gg \beta^*$, TA plasmids persist within singularly infected and co-infected hosts, $n_{TA}{}^* > 0$ & $n_{ITA}{}^* > 0$ (Figure 8B).

Now let us instead consider the invasion of a high-cost TA-encoding plasmid, $c = 0.4$ given $\alpha = 1$ (i.e. a relative vertical fitness within singularly infected host of 0.6). Time series show that as in the low-cost case, high-cost TA plasmids may invade given sufficient horizontal transfer. However, if the rate of horizontal transfer is only modestly greater than its critical threshold, $\beta > \beta^*$, null plasmid and TA plasmid persist at the interior coexistence equilibrium, $n_I{}^* > 0$, $n_{TA}{}^* > 0$ and $n_{ITA}{}^* > 0$. Whereas increased rates of horizontal transfer, $\beta \gg \beta^*$, results in the elimination of hosts that bear only null plasmid (Figure 9).

To summarise, numerical exploration suggests model (2.5) has four positive non-trivial steady states: a null plasmid only equilibrium, $\mathcal{E}_1 = (n_{I_1}^*, 0, 0)$; a TA plasmid only equilibrium $\mathcal{E}_2 = (0, n_{TA_2}^*, 0)$; and two plasmid coexistence equilibria, $\mathcal{E}_3 = (n_{I_3}^*, n_{TA_3}^*, n_{ITA_3}^*)$ and $\mathcal{E}_4 = (0, n_{TA_4}^*, n_{ITA_4}^*)$, the later only permitting null

plasmids to exist within co-infected hosts. In the next section we formalise these numerical findings with analytical results.

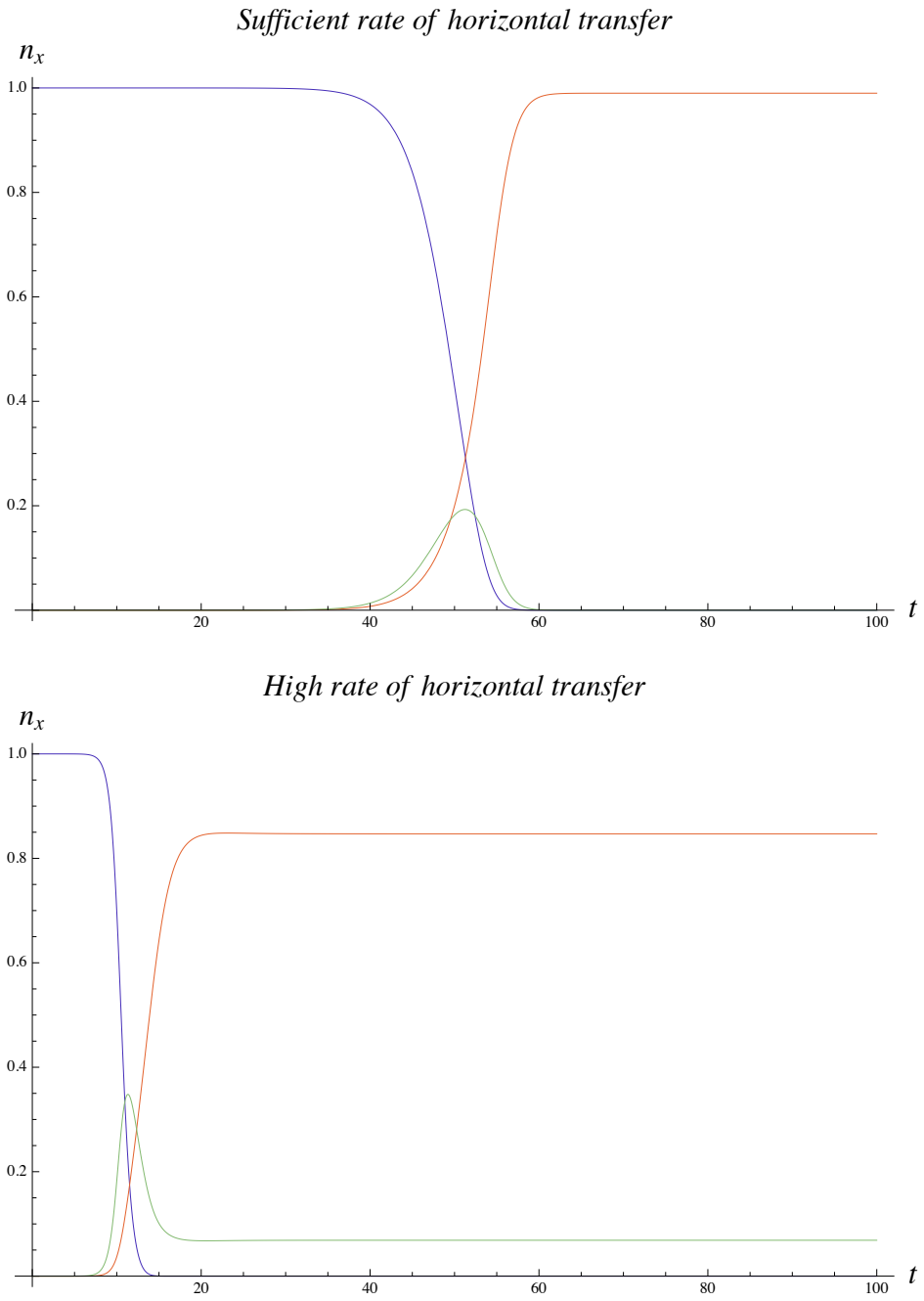

Figure 8 Time series of model (2.5) showing the effect of infectious transfer (β) on the invasion dynamics of an initially rare low-cost TA plasmid. The densities of, n_I, n_{TA}, and, n_{ITA}, in blue, orange and green, respectively. Top: $\beta = 1.5$, invasion and subsequent elimination of null plasmid. Bottom: $\beta = 4.5$, invasion and subsequent elimination of null plasmid in a single-infected state. The remaining parameters were $\alpha = 1$, $c = 0.01$, $\mu = 1$, and initial condition, $n_I(0) = \frac{\alpha}{\mu}$, $n_{TA}(0) = 10^{-8}$ and $n_{ITA}(0) = 0$.

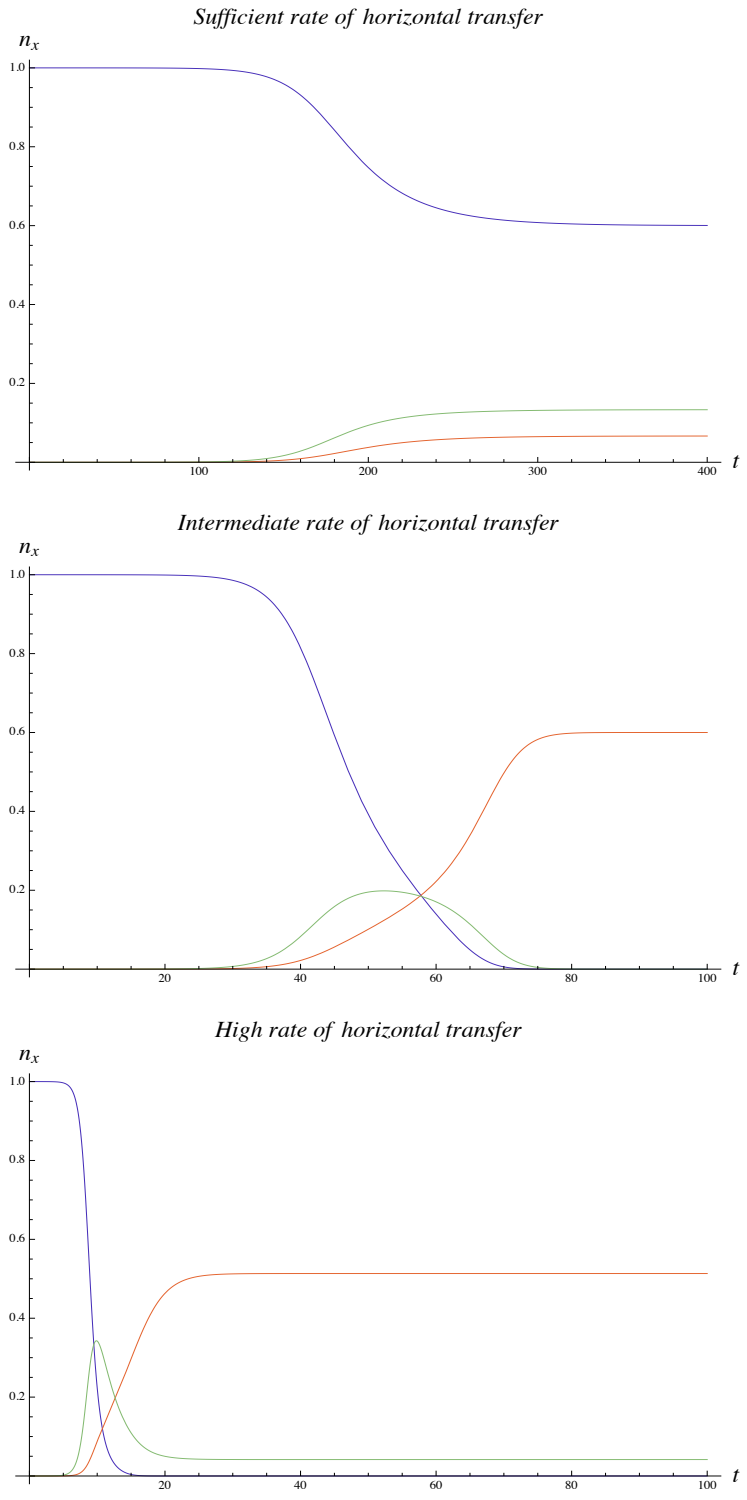

Figure 9 Time series of model (2.5) showing the effect of infectious transfer (β) on the invasion dynamics of an initially rare high-cost TA plasmid. High-cost TA plasmids may not necessarily drive null-plasmids to extinction; instead all three host-types may coexist. This is in contrast to low-cost TAs (see Figure 8). The densities of, n_I, n_{TA}, and, n_{ITA}, in blue, orange and green respectively. Top: $\beta = 1.5$, invasion and subsequent coexistence of all three host-types. Middle: $\beta = 2$, invasion and subsequent elimination of null plasmid. Bottom: $\beta = 4.5$, invasion and subsequent elimination of null plasmid in a single-infected state. The remaining parameters as in Figure 8 except $c = 0.4$.

(ii) Invasion dynamics

We now address the question: assuming co-infection, what conditions permit the invasion of costly TA-encoding plasmids? In the absence of TA plasmids, null plasmids exist at equilibrium, $\mathcal{E}_1 = \left(n_{I_1}^* = \frac{\alpha}{\mu}, 0, 0\right)$. The stability of $\mathcal{E}_1$ can be determined by examining the spectral bound of the Jacobian matrix, $s(\mathbf{J_1})$, of model (2.5). Specifically, $\mathcal{E}_1$ loses stability when $s(\mathbf{J_1}) > 0$. In evaluating the spectral bound, we find the condition for invasion of TA plasmids to be (for derivation see Appendix 5(b)),

$$\alpha^2\beta^2 + 2c^2\mu^2 - \alpha c\mu(\beta + 4\mu) > 0\,. \tag{2.6}$$

Inequality (2.6) is the condition for invasion of an initially rare TA plasmid and agrees with numerical tests (see Appendix 5(h)). By solving for β, an expression describing the critical threshold for horizontal transfer can be derived

$$\beta > \beta^* = \frac{\left(c + \sqrt{(16\alpha - 7c)c}\right)\mu}{2\alpha}. \tag{2.7}$$

Two relationships can be gleaned from critical value, β^*. First, β^* is monotonically decreasing with intrinsic growth rate, α, $\frac{\partial}{\partial\alpha}\beta^* < 0$ given $\alpha \geq c$ (see Appendix 5(c)). That is, as the intrinsic rate of vertical reproduction, α, increases, the required rate of horizontal transfer needed to permit invasion decreases. The equilibrium density of null plasmid-bearing hosts in the absence of TA plasmids, $n_{I_1}^* = \frac{\alpha}{\mu}$, increases with α. Therefore, the required rate of horizontal transfer at equilibrium $\beta n_{I_1}^*$, is satisfied with smaller β. In addition, since cost, c, is modelled as an absolute value, an increase in the intrinsic growth rate, α, reduces the relative cost of the TA complex. Second, β^* is monotonically increasing with c, $\frac{\partial}{\partial c}\beta^* > 0$ given $\alpha \geq c$ i.e. as the cost of encoding a TA complex, c, increases, a greater rate of horizontal transfer is required to permit invasion (Figure 10).

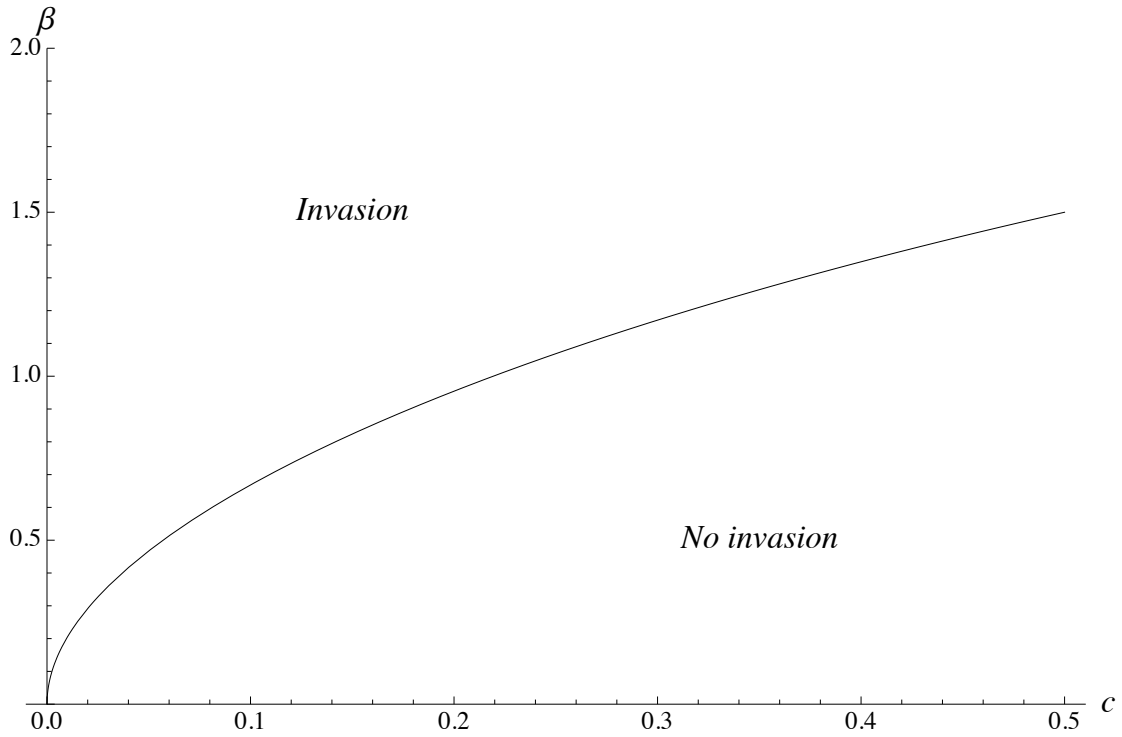

Figure 10. The critical threshold of horizontal transfer, β^*, that permits invasion of TA plasmids (inequality (2.7)). Parameters were $\alpha = 1$ and $\mu = 1$.

Interestingly, within-host competition does not affect inequality (2.6) and therefore extends to any rare mutant that is more costly than the incumbent plasmid (see Appendix 5(d)). That is, *addiction does not change the invasion dynamics of plasmids, but rather alters the evolutionary stability of competing replicons*. We now examine the effect of addiction complexes on plasmid population dynamics.

(iii) Post-invasion dynamics

Inequality (2.6) is the general condition for the invasion of TA plasmids (i.e. the value at which $\mathcal{E}_1 = \left(\frac{\alpha}{\mu}, 0, 0\right)$ loses stability). However, upon invasion the steady state densities n_I, n_{TA} and n_{ITA} converge to one of either equilibrium $\mathcal{E}_2 = \left(0, \frac{\alpha - c}{\mu}, 0\right)$, $\mathcal{E}_3 = \left(n_{I_3}^*, n_{TA_3}^*, n_{ITA_3}^*\right)$ or $\mathcal{E}_4 = \left(0, n_{TA_4}^*, n_{ITA_4}^*\right)$.[4] . We now examine the critical parameter values (i.e. bifurcations) that determine which equilibrium is converged upon.

[4] Analytical results for $\mathcal{E}_3$ were attained using the computer algebra system Mathematica, however, due to the size of the expressions they have been omitted from the text.

We can see that a loss in stability of $\mathcal{E}_1$ (i.e. TA plasmid invasion) results from a gradual increase in horizontal transfer, β (Figure 12A & Figure 13A). In the case of a low-cost TA complex, null plasmids are eliminated and TA plasmids persist within singularly infected hosts, i.e. the system converges to equilibrium $\mathcal{E}_2$ (Figure 12). In the case of a high-cost TA complex the system converges to the coexistence equilibrium $\mathcal{E}_3$ with a gradual increase in β. However, with greater increases in β, null plasmids are eliminated and TA plasmids persist within singularly infected hosts as the system converges to $\mathcal{E}_2$ (Figure 13).

Equilibrium $\mathcal{E}_2$ is the steady state at which TA plasmids persist solely within singularly infected hosts, while null plasmids are absent $(0, n_{TA_2^*} = \frac{\alpha - c}{\mu}, 0)$. The threshold at which $\mathcal{E}_2$ loses stability can be determined by evaluating $s(\mathbf{J}) > 0$. In doing so, we are left with two bounding conditions (assuming $c < 4\alpha/5$) for the stability of $\mathcal{E}_2$ which can be expressed as a bounded threshold of horizontal transfer, β (see Appendix 5(e) for derivation),

$$\frac{c\mu}{\alpha - c} < \beta < 4\mu\,. \tag{2.8}$$

The lower bound of stability, ${\beta_2}^* = \frac{c\mu}{\alpha - c}$, means that horizontal transfer at equilibrium, $\beta n_{TA_2^*}$, must outweigh the cost of the TA complex, $\beta n_{TA_2^*} > c$, and is similar to what was derived for plasmid invasion in a single plasmid system (see inequality (2.2)). The upper bound of stability, ${\beta_3}^* = 4\mu$, is the condition that permits the invasion of null plasmids. Due to perfect replication incompatibility, symmetric segregation and post-segregational killing, invading null plasmids must reproduce by horizontal transfer alone. Therefore, the null plasmid is only able to persist within co-infected hosts with its sole means of reproduction being conjugation.

Upon the establishment of a TA plasmid-only population, the conditions required for re-establishment of a null plasmid-only population becomes severely restricted. This is due to an asymmetry between the stability of $\mathcal{E}_1$ and $\mathcal{E}_2$. The critical threshold, ${\beta_1}^*$, exists when approached from $\mathcal{E}_1$ and not from $\mathcal{E}_2$ (hysteresis). Instead, $\mathcal{E}_2$ loses stability at the more restrictive critical threshold, ${\beta_2}^*$, which occurs when the rate of

horizontal transfer is insufficient to offset the cost of the TA complex. Importantly, this finding accounts for the empirical observation that TA plasmids are resistant to invasion by incompatible plasmids (T. Naito *et al.*, 1995; Y. Naito *et al.*, 1998; Cooper & Heinemann, 2000). This is in contrast to previous models that fail to predict this hysteresis effect (Mochizuki *et al.*, 2006; Cooper *et al.*, 2010; Rankin *et al.*, 2012).

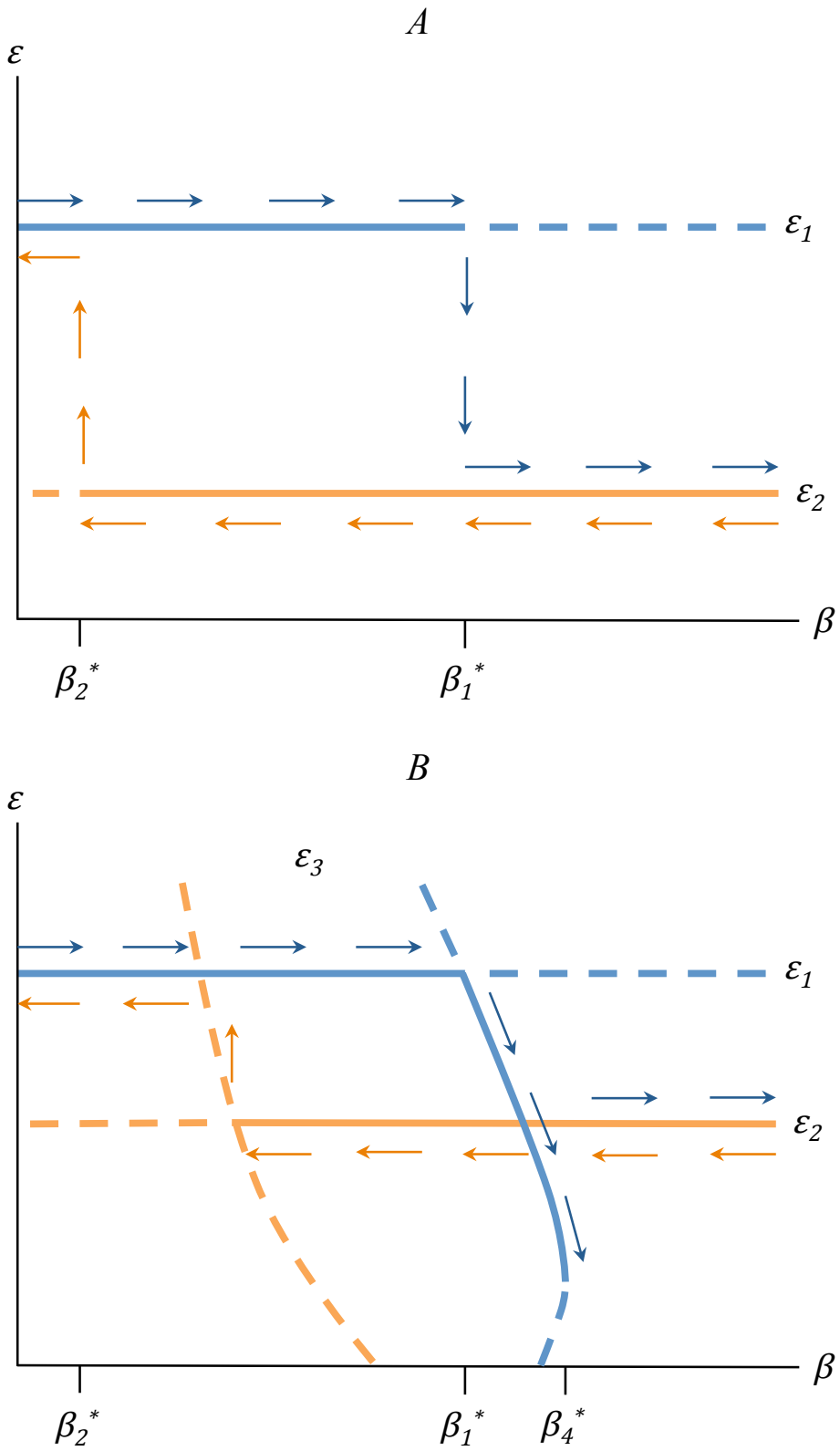

Figure 11 A schematic representation of equilibria $\mathcal{E}_1$ and $\mathcal{E}_2$ by horizontal transfer, β, showing hysteresis. The null plasmid-only steady-state, $\mathcal{E}_1$, loses stability with increasing β (blue arrows) at β_1^*. Upon invasion, the TA plasmid-only state, $\mathcal{E}_2$, becomes stable. However, with decreasing β (orange arrows) $\mathcal{E}_2$ loses stability at β_2^*. This asymmetry in stability is due to post-segregational killing, where TA plasmids resist invasion by killing segregants of co-infected cells. (A) modest TA cost (B) high TA cost.

Equilibrium $\mathcal{E}_3 = \left(n_{I_3}^*, n_{TA_3}^*, n_{ITA_3}^*\right)$ is the steady state at which all bacterial genotypes coexist and is only stable when the TA complex confers a considerable cost. This cost reduces the rate of cell division in co-infected hosts and in turn the rate of post-segregational killing, $(\alpha - c)n_{ITA}$. The coexistence equilibrium, $\mathcal{E}_3$, arises when TA plasmid is able to invade but unable to purge the population of null plasmid via post-segregational killing. Determining the stability of $\mathcal{E}_3$ is therefore crucial for understanding plasmid-plasmid competition.

An analytical result for $\mathcal{E}_3$ was attained using the computer algebra system Mathematica. The solution $\mathcal{E}_3$ actually consists of two related solutions in the form $x \pm \sqrt{y}$. Due to the analytical complexity of $\mathcal{E}_3$, determining bifurcations using conventional eigenvalue techniques proved intractable. Fortunately, a heuristic for the stability of $\mathcal{E}_3$ was identified by numerical exploration; namely, $\mathcal{E}_3$ is stable when its solution *exists,* i.e. when the equilibrium densities of each cell type are positive and real (see Figure 12C & Figure 13C). By examining solution $n_{ITA_3}^*$ it can be shown that $\mathcal{E}_3$ becomes complex, and therefore loses stability, when (see Appendix 5(f) for derivation)

$$4\alpha^2(3\beta - 2\mu)(\beta + 2\mu) + c^2\beta(7\beta + 16\mu) - 20\alpha c\beta(\beta + 2\mu) > 0. \quad (2.9)$$

Rearranging (2.9) for β yields the following critical threshold for horizontal transfer

$$\beta > {\beta_4}^* = \frac{-4\mu(\alpha-2c)(2\alpha-c)+8\sqrt{(\alpha-c)(2\alpha-c)(2\alpha^2-2\alpha c+c^2)\mu^2}}{(6\alpha-7c)(2\alpha-c)}. \quad (2.10)$$

The threshold, ${\beta_4}^*$, represents the amount of horizontal transfer required to ensure that upon invasion of the TA plasmid, the null plasmid is purged (it may still persist in a co-infected state but it is unable to persist as a "free-living" plasmid). If the rate of horizontal transfer is less than ${\beta_4}^*$, then the effect of within-host competition is muted due to insufficient levels of co-infection.

Since cost is modelled generally as a reduction in host fecundity, it may capture any process that reduces the birth rate of the host, such as traits that cause bacteriostasis.

This implies that plasmid competition by post-segregational killing is at odds with bacteriostasis and that the simultaneous presence of both phenotypes reduces the ability to kill competing plasmids. This is important when considering plasmid-level competition. If segregational loss is coupled with post-segregation killing, one would expect general mechanisms to exist that attempt to salvage plasmid miss-segregation. Plasmid-rescue, where plasmids with depressed copy-numbers inhibit cell division of their host until there are sufficiently many replicon copies to faithfully segregate to both daughter cells (Pimentel *et al.*, 2005; 2014), may be such a system. However, its evolutionary interpretation as a plasmid competition defence mechanism has not yet been considered (see discussion for more).

The final equilibrium, $\mathcal{E}_4$ $\left(n_{I_4}^* = 0, n_{TA_4}^*, n_{ITA_4}^*\right)$, is the steady-state at which null plasmids may only exist within cells co-infected by TA plasmids. It arises when TA plasmids are able to invade but unable to purge the population of null plasmids due to high rates of horizontal transfer. In this scenario, null plasmids persist by horizontal transfer alone as cell division results in death due to PSK. Equilibrium $\mathcal{E}_4$ has two solutions which take the form $x \pm \sqrt{y}$. The subtractive solution can be discarded as either $n_{TA_4}^*$ or $n_{ITA_4}^*$ is negative given positive parameter values leaving us with the additive solution as the only solution of interest (see Appendix 5(g) for details). The threshold at which $\mathcal{E}_4$ loses stability can be determined by evaluating $s(\mathbf{J}) > 0$. In doing so, we are left with the following threshold of horizontal transfer, β.

$$\beta > \beta_3{}^* = 4\mu. \tag{2.11}$$

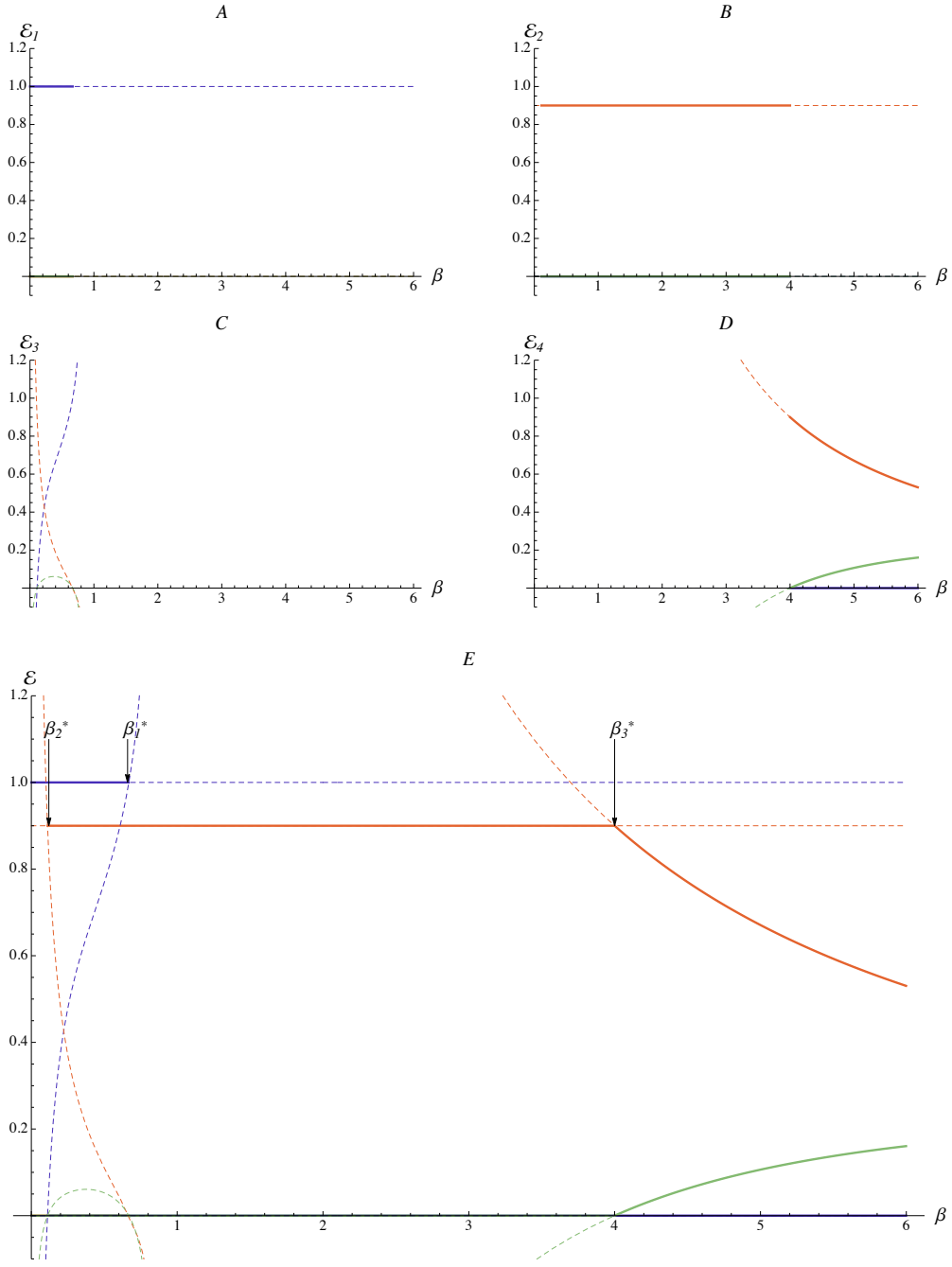

Figure 12. A continuation of equilibrium densities n_I^*, n_{TA}^* and n_{ITA}^* by horizontal transfer, β, assuming the TA complex confers a modest cost, $c = 0.1$. At a modest cost, $c = 0.1$, the host-coexistence equilibrium, $\mathcal{E}_3$, is unstable (C), meaning TA plasmids drive singularly infected hosts bearing null-plasmid to extinction upon invasion. Densities n_I^*, n_{TA}^* and n_{ITA}^* are in blue, orange and green respectively. Solid lines indicate when a positive equilibrium is stable while dashed lines indicate when an equilibrium is unstable. A, B, C and D are continuations of $\mathcal{E}_1$, $\mathcal{E}_2$, $\mathcal{E}_3$ and $\mathcal{E}_4$ respectively. E is a superposition of A, B, C and D with bifurcations present in this parameter-space; β_1^*, β_2^* and β_3^* indicated. Remaining parameters values were $\alpha = 1$ and $\mu = 1$. Stability was determined evaluating the spectral bound of the Jacobian, $s(\mathrm{J})$, at each fixed point. Specifically, a point is unstable when $s(\mathrm{J}) > 0$. These results agreed with numerical integration along the line-space of each fixed point.

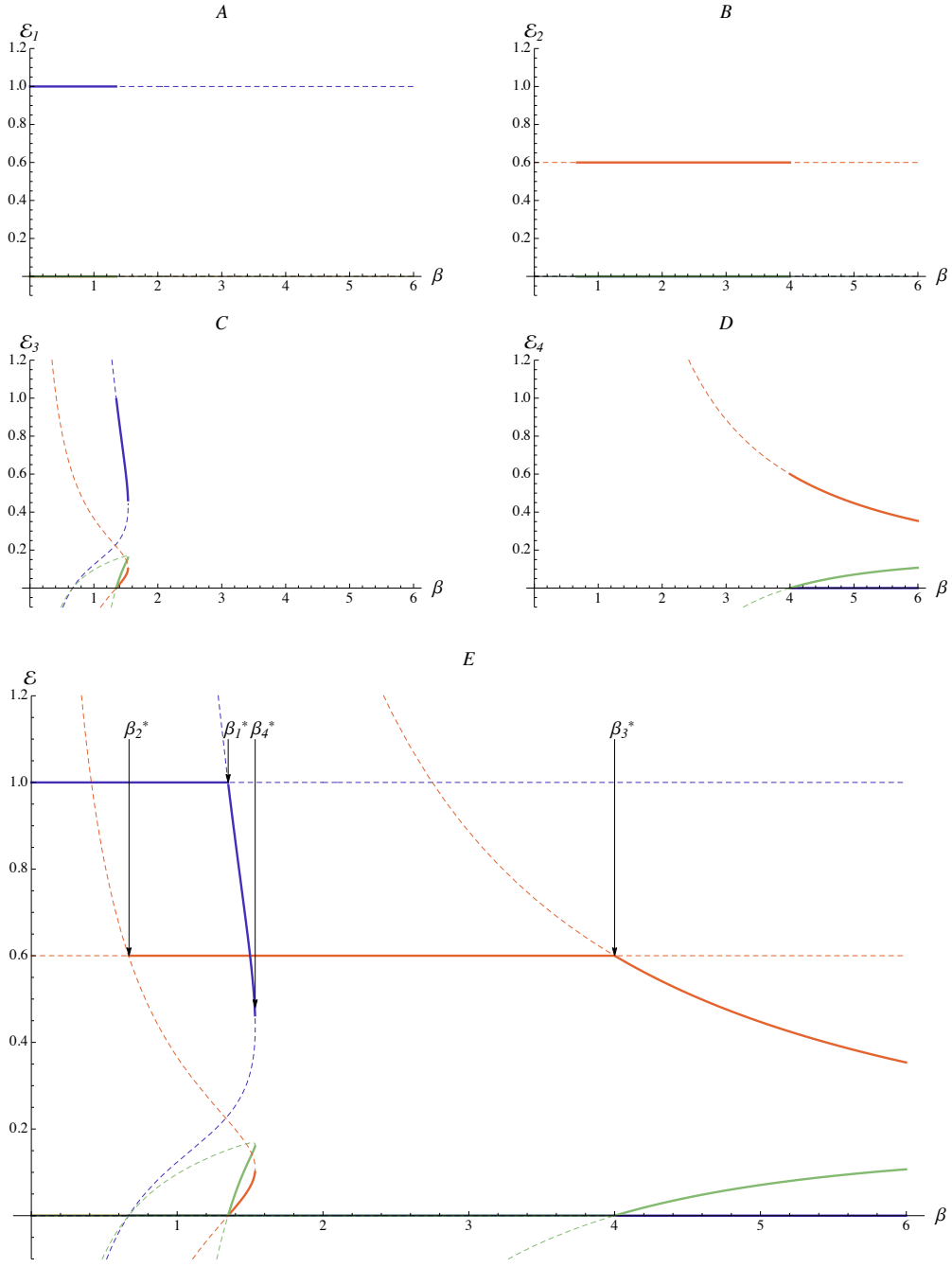

Figure 13. A continuation of equilibrium densities n_I^*, n_{TA}^* and n_{ITA}^* by horizontal transfer, β, assuming the TA complex confers a high cost, $c = 0.1$. At a high cost, $c = 0.4$, the host-coexistence equilibrium, $\mathcal{E}_3$, is locally stable (C), meaning TA plasmids may not necessarily drive singularly infected hosts bearing null-plasmid, n_I, to extinction. Instead, extinction of n_I now depends of the stability of $\mathcal{E}_3$, specifically, bifurcation β_4^*. Densities n_I^*, n_{TA}^* and n_{ITA}^* are in blue, orange and green respectively. Solid lines indicate when a positive equilibrium is stable while dashed lines indicate when an equilibrium is unstable. A, B, C and D are continuations of $\mathcal{E}_1$, $\mathcal{E}_2$, $\mathcal{E}_3$ and $\mathcal{E}_4$ respectively. E is a superposition of A, B, C and D with bifurcations present in this parameter-space, β_1^*, β_2^*, β_3^* and β_4^*, indicated. Remaining parameters values were $\alpha = 1$ and $\mu = 1$. Stability was determined evaluating the spectral bound of the Jacobian, $s(\mathbf{J})$,

at each fixed point. Specifically, a point is unstable when $s(\mathbf{J}) > 0$. These results agreed with numerical integration along the line-space of each fixed point.

In summary, the critical thresholds β_1^*, β_2^*, β_3^* and β_4^* constitute the bifurcations of model (2.5) (Figure 14). Threshold β_1^* is where TA plasmids may invade a population of null plasmid-bearing bacteria. This is a general condition for the invasion of any distinct co-infecting plasmid replicon. Upon invasion, the system will converge to either $\mathcal{E}_2$ (TA plasmid only hosts), $\mathcal{E}_3$ (null plasmid only, TA plasmid only and co-infected hosts) or $\mathcal{E}_4$ (TA plasmid-only and co-infected hosts) depending on the rate of horizontal transfer and the cost of the TA complex. Where horizontal transfer is sufficiently fast (i.e. β being modestly greater than β_1^*) and a reduction in vertical reproduction is in the order of empirically derived estimates (i.e. low cost, c), TA plasmids invade and eliminate null plasmids (i.e. $\mathcal{E}_2$ becomes stable). Equilibrium $\mathcal{E}_2$ is resistant to invasion by null plasmids and loses stability at the restrictive thresholds β_2^* and β_3^*. At β_2^*, the rate horizontal transfer is insufficient and unable to overcome the cost of the TA complex. Whereas at β_3^* horizontal transfer is sufficiently high that null plasmids invade and persist owing to horizontal transfer alone. Moreover, if the TA complex drastically reduces the rate of vertical reproduction (i.e. high cost, c) then so are incidences of PSK. In this situation, TA plasmids are able to invade but unable to eliminate the null plasmid and thus all three genotypes coexist (i.e. $\mathcal{E}_3$ becomes stable). The reduced killing of null plasmids, due to the retardation of cell division in co-infected hosts, can be overcome by increasing the rate of co-infection (i.e. the rate of horizontal transfer). At threshold β_4^*, horizontal transfer is sufficiently high to permit the elimination of null plasmid.

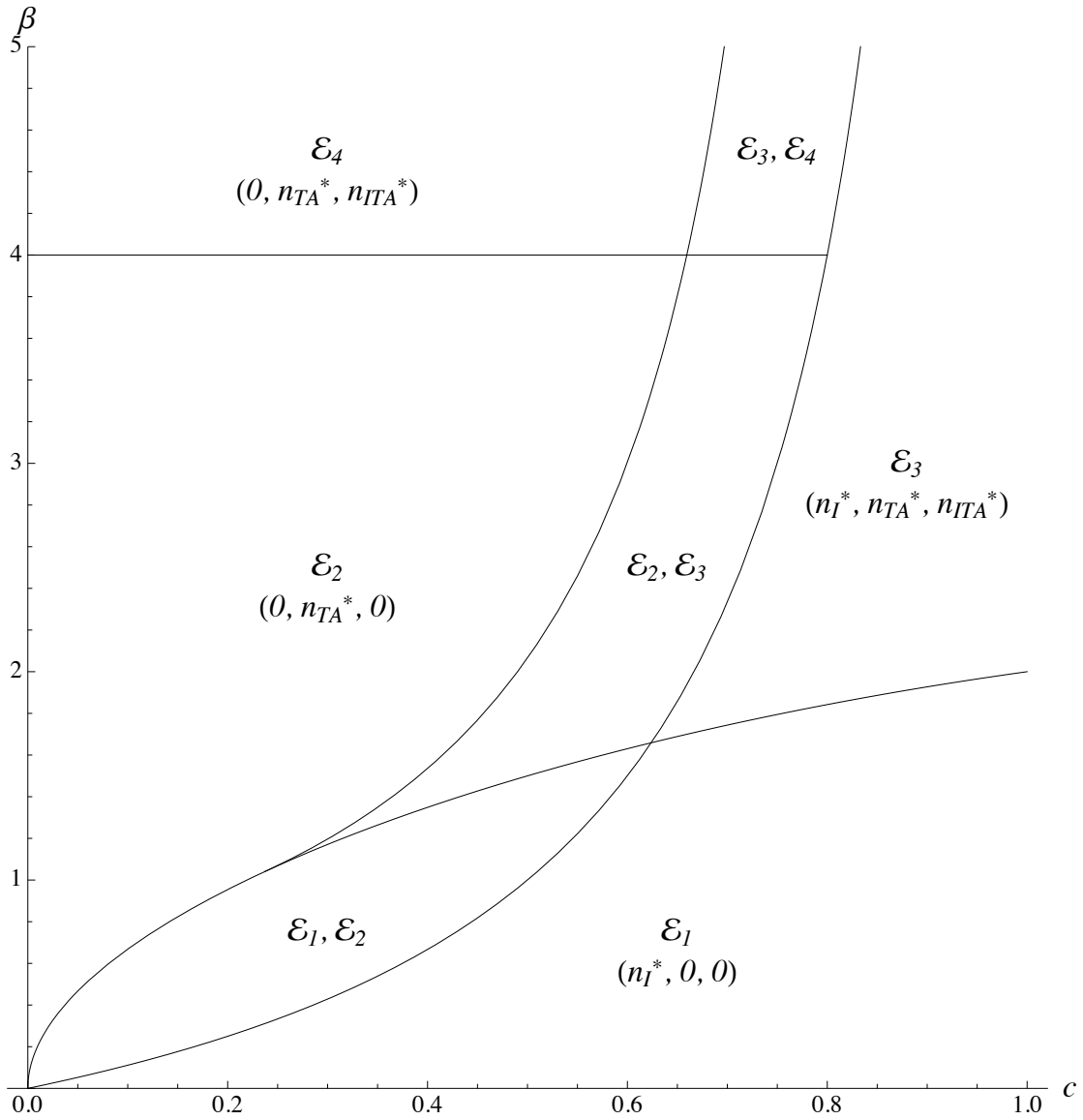

Figure 14. Bifurcation diagram of model (2.5) showing the relationship between TA cost, c, and horizontal transfer, β. Co-existing stable points arise through differences in initial conditions. Parameters are $\alpha = 1$ and $\mu = 1$.

3 DISCUSSION

Plasmid fitness derives from a complex assortment of interactions between not only the host, but a range of competing intra-cellular replicons. Any plasmid mutant arising in a population of plasmid bearing hosts will have to outcompete its siblings. We explored the dynamics of toxin-antitoxin mediated competition between co-infecting plasmids and showed that addiction complexes are adaptive to lower-level replicons such as plasmids by decreasing the fitness of plasmids that compete for vertical reproduction. The following four key findings were made by our analysis.

Firstly, we show that plasmid borne toxin-antitoxin systems can invade even if they reduce cell fitness. Previous models have used environmental structure to explain how a mal-adaptive trait like addiction may spread in bacterial populations (Mochizuki *et al.*, 2006; Rankin *et al.*, 2012). These models assume that resources freed by cell death preferentially benefit related cells (altruistic benefit). The implication is that, segregational loss of a plasmid is offset by increased host fecundity, implying host-killing provides an indirect reproductive benefit to the focal cell (i.e. host-adaption). In agreement with empirical observation, we find that a kin-advantage (i.e. environmental structuring) is not required if co-infection is taken into account.

Secondly, we derived analytical expressions that describe how plasmid incompatibility and addiction mediate within-host competition between plasmids. The dynamics of addiction-mediated competition were surprisingly complex and were dependent on the rate of vertical reproduction as well as the cost of addiction. When the rate of horizontal transfer is low (i.e. infrequent co-infection) and the cost of the addiction complex is high (i.e. supressed cell growth rate), plasmids bearing addiction complexes were unable to purge incompatible plasmids that lack immunity. Instead, all three cell states (null plasmid cells, TA plasmid cells and co-infected cells) could co-exist owing to the infrequency of plasmid-killing. However, if the addiction complex did not cause an excessive reduction in cell fecundity, addiction bearing plasmids were able to purge plasmids lacking immunity.

Thirdly, and in agreement with empirical observation, our model predicts that plasmids bearing addiction complexes are resistant to invasion from plasmids lacking immunity, but not vice versa. This means that the condition for re-invasion of a plasmid lacking immunity is much more stringent than that of the establishment of the TA plasmid i.e. hysteresis.

Fourthly, and perhaps most interestingly, our model suggests that plasmids bearing addiction complexes can outcompete plasmids lacking immunity even if the incumbent plasmid confers a significant adaptive advantage to the cell. This may suggest that plasmid-borne adaptive traits like antibiotic resistance determinants could potentially be purged by incompatible plasmids bearing addiction complexes.

(a) Predicting plasmid success

Predicting the success of certain plasmid genotypes is of central importance for understanding the movement of genes in bacterial populations. Most *in vitro* studies of plasmid population biology assume plasmids reproduce in a single genotypic background free of competing elements. However, *in situ*, bacteria continually receive and transmit conjugative plasmids, yielding a combinatorially large set of potential genetic backgrounds in which to reproduce. We examined plasmid transmission in two distinct host backgrounds, naïve plasmid-free cells and cells bearing an incompatible plasmid, and found that co-infection radically altered the calculus of plasmid fitness. Specifically, successful plasmid transmission not only relied upon horizontal transfer to new hosts but the ability to vertically transmit in the presence of competing replicons. The classical result for obligate infectious parasites is that parasite maintenance is the sum of vertical and horizontal reproduction (Lipsitch *et al.*, 1995). Co-infection greatly increases the complexity of plasmid fitness where plasmid success depends on the genetic composition (i.e. presence or absence of competing elements) of the receiving population. Our model suggests that plasmid fitness must be viewed in aggregate across all encountered genetic backgrounds. For example, in the absence of competing elements, plasmid fitness may be optimised by host adaptive traits, whereas, in the presence of competing elements, competition determinants, like toxin-antitoxin systems, may greatly enhance relative plasmid fitness. Mean plasmid fitness will ultimately depend on the importance of reproducing

in each genetic background (i.e. the frequency of each genetic background in the population). This notion of plasmid fitness stands in contrast to current models of plasmid population biology that assume fitness is fully described by the traits borne by the plasmid and its relationship to a single host background.

(b) Competitive counter-measures

The dynamics observed in our model suggest the existence of a number of co-evolutionary relationships between plasmid traits. Competition mediated by toxin-antitoxin systems relies on three features:

1. co-localisation of competing replicons;
2. frequent (non-random) segregation of replicons e.g. reproductive conflict; and
3. differential susceptibility to the action of the toxin.

The following are potential counter measures that either resolve or mitigate the effect of toxin-antitoxin mediated competition between plasmid replicons.

(i) Acquisition of immunity

Perhaps the simplest counter measure is to acquire resistance to the toxin (Cooper & Heinemann, 2005). Since toxin-antitoxin systems typically comprise closely linked toxin and antitoxin loci, it is likely the entire complex is gained during introgression. If the recipient replicon is in reproductive conflict, both traits may be maintained, however, if host-killing is not adaptive to the new replicon (e.g. a chromosome) one would assume purifying selection to gradually disrupt toxin activity but maintain immunity if competition is persistent at other levels.

(ii) Plasmid-rescue (bacteriostasis)

Plasmid-rescue is when cell division is arrested whenever the replicon bearing the determinant exists at relatively too few copies to ensure faithful segregation to both daughters of cell division (Pimentel *et al.*, 2005; 2014). The resulting effect is that plasmids bearing rescue loci have increased vertical inheritance stability. The differentiating characteristic of plasmid rescue compared to persistence is that bacteriostasis is dependent on the gene dosage of the toxin-antitoxin locus and the reproductive stage of the cell (for more details see Chapter 1). Plasmid-rescue may present a general mechanism to counter post-segregational killing brought about by reproductive conflicts. At too few gene copies, e.g. when in the presence of a

competing replicon (co-infected cell), plasmid-rescue will halt cell reproduction and upregulate replication initiating proteins preventing segregation of competing plasmids. This strategy is unlikely to be stable in the long term, as repeated bouts of bacteriostasis presumably have an appreciable effect on plasmid fitness, however, it may provide a general defence against post-segregational killing.

(iii) Chromosomal integration

Competition mediated by toxin-antitoxin systems relies on the segregation of replicons at cell division, which in the case of plasmids occurs actively through plasmid partitioning mechanisms. Any mechanism that reduces the rate of segregation between competing replicons (i.e. decreases incompatibility) would reduce the effect of post-segregational killing. Integration into chromosomal replicons, or more generally replicons that are not in reproductive conflict with the antagonist element, would decrease the rate of segregation by transferring replication control to an element that is not in reproductive conflict with the antagonistic replicon. Although introgression into chromosomal replicons does not necessarily destroy incompatibility, for example F plasmid can not be maintained in the HFR cell lines unless an alternative origin is present (Hiraga, 1976), competing elements that segregate from chromosomal replicons are unable to reproduce indefinitely.

(iv) Relaxation or diversification of replication control

Plasmid-borne regulatory molecules act to control plasmid replication, where activators bind the origin of replication, attracting DNA polymerase, while inhibitors interfere with the activity of the activator. The copy-number of a plasmid, or the average number of plasmid molecules per cell, is a function of the kinetic properties of these various regulatory molecules. Mutations that increase *cis*-acting activation or reduce binding affinities of *trans*-acting repressors would reduce the rate of segregation. Overtime, this process could potentially lead to diversification of replication origins i.e. *oriV* speciation. As a result, one may observe genomic correlations between replication origin and toxin-antitoxin alleles.

(v) Surface and entry exclusion

Surface and entry exclusion determinants reduce the incidents of co-infection by inhibiting the entry of foreign plasmids (for a review see Garcillán-Barcia & la Cruz,

2008). Surface and entry exclusion determinants may provide a general mechanism to reduce the co-location of competing replicons. Again, as a result, one may observe genomic correlations between replication origin and surface/entry exclusion alleles.

(c) Plasmid virulence

We found that plasmid virulence is dependent on the frequency of co-infection. In monoculture, virulence can be approximated by plasmid cost-of-carriage, as death by post-segregational killing is negligible when miss-segregation is rare. However, in bi-culture, virulence is best approximated by rates of co-infection and subsequent post-segregational killing. In this case, cell death is incidental to plasmid death and can be viewed as collateral damage. Interestingly, this creates the peculiar situation where chromosomal-borne toxin-antitoxin systems are adaptive if their antitoxin provides immunity to a plasmid-borne toxin when competition is prevalent (Chapter 2; Cooper & Heinemann, 2005). Quantifying virulence, as a result of competition and post-segregational killing, becomes significantly more complex than in the case where virulence is constant and time invariant. In our model, aggregate virulence is proportional to the integral of the co-infected state and may vary in time depending on the prevalence of each plasmid genotype. This suggests that in some cases empirical estimates of plasmid cost are underestimated.

(d) Future work

Above, we have suggested 5 counter-measures to toxin-antitoxin mediate competition in plasmids. These counter-measures are eminently testable by a series of plasmid competition experiments similar to those developed by (Cooper & Heinemann, 2000).

Our model makes the simplifying assumption that vertical and horizontal reproduction between plasmid genotypes is equitable. Future work may wish to examine the relationship between more complex types of within-host plasmid competition such as *trans*-acting repressors and *cis*-acting activators of replication (Paulsson, 2002), horizontal transfer interference between competing mobile elements (Dionisio *et al.*, 2002) and surface and entry exclusion determinants. However, such models will most likely require more complex modelling techniques such as nested models (Mideo *et al.*, 2008) and are less likely to yield analytical insights.

Our model assumes the maximal number of plasmids that can co-infect a cell is two, however, in nature no such restriction exists. Recent modelling techniques that more generally account for arbitrary numbers of intracellular parasites (Sofonea *et al.*, 2015) may be of use for studies of plasmid evolution and population biology.

(e) Concluding remarks

Formulating bacterial genomes as communities of interacting replicons may provide insights into the near-term evolution of microbial communities. Our model suggests, that in some situations, the ecological dynamics between genetic elements is a better predictor of bacterial evolution than models focusing on cellular adaptation alone. Moreover, our work highlights the importance of viewing adaptation at the level of the replicon. In microbial communities, Darwinian Individuals are perhaps best thought of as assortments of traits that faithfully transmit together by physical linkage or co-transmission.

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5 APPENDIX

(a) Derivation of inequality (2.4)

TA plasmids may invade from rare provided their per capita rate of change at the coexistence equilibrium $\mathcal{E}_2 = (n_{F_2}^*, n_{I_2}^*)$ is greater than zero

$$\frac{dn_{TA}}{dt}\frac{1}{n_{TA}} > 0. \tag{5.1}$$

We know that at the interior coexistence equilibrium $\mathcal{E}_2$ the per capita rate of change of n_I is zero,

$$\frac{dn_I}{dt}\frac{1}{n_I} = a - \mu\left(n_{F_2}^* + n_{I_2}^*\right) + \beta n_{F_2}^* - as - x \equiv 0, \tag{5.2}$$

and that the per capita rate of change of n_{TA} at $\mathcal{E}_2$ is

$$\frac{dn_{TA}}{dt}\frac{1}{n_{TA}} = a - \mu\left(n_{F_2}^* + n_{I_2}^* + n_{TA}\right) + \beta n_{F_2}^* - as - x - c. \tag{5.3}$$

By using equivalence (5.2), equation (5.3) simplifies to

$$\frac{dn_{TA}}{dt}\frac{1}{n_{TA}} = -\mu n_{TA} - c. \tag{5.4}$$

Now assuming the initial density of TA plasmids is small i.e. $n_{TA} \approx 0$, a first approximation for the invasion of an initially rare TA plasmid at $\mathcal{E}_2$ is

$$c < 0. \tag{5.5}$$

(b) Derivation of inequality (2.6)

Stability is assessed by examining the spectral bound of the Jacobian matrix, $s(\mathbf{J})$, of model (2.5) evaluated at ${n_I}^* = \frac{\alpha}{\mu}$ and ${n_{TA}}^* = {n_{ITA}}^* = 0$, where

$$s(\mathbf{J}) = \mathrm{Re}\left(\frac{-\alpha(\beta+4\mu)\pm\sqrt{9\alpha^2\beta^2-9\alpha^2\beta\mu-8\alpha c\beta\mu+16\alpha^2\mu^2-32\alpha c\mu^2+16c^2\mu^2}}{4\mu}\right) \quad (5.6)$$

The equilibrium loses stability when $s(\mathbf{J}) > 0$. Because the death rate, μ, is constrained to be positive, the condition for invasion can be simplified by rewriting the numerator of equation (5.6) in the form $-A \pm \sqrt{A^2 - B} > 0$ where $A = \alpha(\beta + 4\mu)$ and $B = \alpha^2\beta^2 + 2c^2\mu^2 - \alpha c\mu(\beta + 4\mu)$. Since $A > 0$, if $B > 0$ then $s(\mathbf{J}) > 0$, thus, the condition for invasion is

$$\alpha^2\beta^2 + 2c^2\mu^2 - \alpha c\mu(\beta + 4\mu) > 0 \quad (5.7)$$

(c) Monotonicity of equation (2.7) with respect to α and c

The critical threshold for invasion of TA-encoding plasmid is

$$\beta > \beta^* = \frac{\left(c + \sqrt{(16\alpha - 7c)c}\right)\mu}{2\alpha} \quad (5.8)$$

If the partial derivative of some function, $f(x)$, with respect to x, is greater than zero then f is an increasing function of x. Analogously, if the partial derivative is less than zero then f is a decreasing function of x.

$$\frac{\partial\beta^*}{\partial\alpha} = -\frac{c\left(8\alpha - 7c + \sqrt{(16\alpha - 7c)c}\right)\mu}{2\alpha^2\sqrt{(16\alpha - 7c)c}} \quad (5.9a)$$

$$\frac{\partial\beta^*}{\partial c} = \frac{\left(1 + \frac{8\alpha - 7c}{\sqrt{(16\alpha - 7c)c}}\right)\mu}{2\alpha} \quad (5.9b)$$

Hence, given $\alpha \geq c$, $\frac{\partial\beta^*}{\partial\alpha} < 0$ and $\frac{\partial\beta^*}{\partial c} > 0$.

(d) Invasion is independent of post-segregational killing

In model (2.5) post-segregational killing results in the death of null plasmids that segregate during cell division of co-infected cells. This effect is captured solely by the partial derivative $\partial n_I/\partial n_{ITA}$. We can conclude that post-segregational killing does not affect invasion if the trace and determinant of the Jacobian evaluated at $\mathcal{E}_1$ is *not* a function of $\partial n_I/\partial n_{ITA}$. Hence, instead of analysing a new model we can simply examine the Jacobian of model (2.5).

The Jacobian of model (2.5) evaluated at $\mathcal{E}_1 = \left(\frac{\alpha}{\mu}, 0, 0\right)$ is

$$\mathbf{J_1} = \begin{pmatrix} \frac{\partial n_I}{\partial n_I} & \frac{\partial n_I}{\partial n_{TA}} & \frac{\partial n_I}{\partial n_{ITA}} \\ 0 & \frac{\partial n_{TA}}{\partial n_{TA}} & \frac{\partial n_{TA}}{\partial n_{ITA}} \\ 0 & \frac{\partial n_{ITA}}{\partial n_{TA}} & \frac{\partial n_{ITA}}{\partial n_{ITA}} \end{pmatrix} = \begin{pmatrix} a & b & c \\ d & e & f \\ g & h & i \end{pmatrix}. \tag{5.10}$$

The trace, $tr(\mathbf{J_1})$, is the sum of diagonal components, $tr(\mathbf{J_1}) = a + e + i$. The determinant, $det(\mathbf{J_1})$, evaluated at $\mathcal{E}_1$ is $det(\mathbf{J_1}) = a(ei - fh)$. As $tr(\mathbf{J_1})$ and $det(\mathbf{J_1})$ are not functions of $\partial n_I/\partial n_{ITA}$ we can conclude that post-segregational killing does not affect condition (2.6), meaning condition (2.6) is a general condition for the invasion of an incompatible plasmid assuming co-infection.

(e) Derivation of inequality (2.8)

The spectral bound of the Jacobian, $s(\mathbf{J_2})$, at $\mathcal{E}_2 = (0, \frac{\alpha - c}{\mu}, 0)$ is,

$$s(\mathbf{J}) = \max\left(\frac{(\alpha - c)(\beta - 4\mu)}{2\mu}, -(\alpha - c), \frac{c\mu - \beta(\alpha - c)}{\mu}\right). \tag{5.11}$$

$\mathcal{E}_2$ loses stability when $s(\mathbf{J_2}) > 0$. Assuming $\alpha > c$ and $\mu > 0$, we are left with two distinct conditions for stability of $\mathcal{E}_2$:

$$\frac{c\mu - \beta(\alpha - c)}{\mu} > 0\,, \tag{5.12a}$$

$$\beta - 4\mu > 0. \tag{5.12b}$$

From these, two critical thresholds (bifurcations) of horizontal transfer exist, ${\beta_2}^* = \frac{c\mu}{\alpha - c}$ and ${\beta_3}^* = 4\mu$.

(f) Derivation of inequality (2.9)

An analytical solution for $\mathcal{E}_3 = (n_{I_3}^*, n_{TA_3}^*, n_{ITA_3}^*)$ was attained but due to its complexity the spectral bound of the Jacobian evaluated at $\mathcal{E}_3$ could not be evaluated analytically. However, by exploring the stability of $\mathcal{E}_3$ numerically the following heuristic for stability was observed: $\mathcal{E}_3$ *is stable when it exists (i.e. when solutions* $n_{I_3}^*$, $n_{TA_3}^*$ and $n_{ITA_3}^*$ *are real and positive).* Therefore, stability can be determined by assessing when:

1. $\mathcal{E}_3$ becomes complex i.e. when the discriminant becomes negative; and,
2. $\mathcal{E}_3$ has a negative component i.e. negative $n_{I_3}^*$, $n_{TA_3}^*$ and $n_{ITA_3}^*$.

Deriving an analytical expression for stability can be simplified by observing that at $\mathcal{E}_3$ solutions $n_{I_3}^*$, $n_{TA_3}^*$ and $n_{ITA_3}^*$ become complex simultaneously and $n_{TA_3}^*$ and $n_{ITA_3}^*$ develop negative solutions simultaneously. Therefore, a single equilibrium solution, $n_{ITA_3}^*$ (the most tractable), can be used for the analysis.

The solution of $n_{ITA_3}^*$ at $\mathcal{E}_3$ is,

$$n_{ITA_3}^* = \frac{\beta X \pm \sqrt{\delta Y}}{Z}, \tag{5.13a}$$

where,

$$X = 2c^2\beta\mu(2\beta + 5\mu) + 2\alpha^2(\beta + 2\mu)(\beta^2 + 4\beta\mu - 4\mu^2) \tag{5.13b}$$
$$-\alpha c(\beta + 2\mu)(\beta^2 + 14\,\beta\mu - 4\mu^2),$$

$$Y = 4\alpha^2(3\beta - 2\mu)(\beta + 2\mu) + c^2\beta(7\beta + 16\mu) - 20\alpha c\beta(\beta + 2\mu), \tag{5.13c}$$

$$Z = (2\alpha - c)\beta^3(\beta + 2\mu)^2, \tag{5.13d}$$

$$\delta = -\beta^2(\alpha\beta^2 - 2(2\alpha - c)\mu^2)^2. \tag{5.13e}$$

(i) The condition at which $\mathcal{E}_3$ becomes complex

The steady state of co-infected cells at $\mathcal{E}_3$, $n_{ITA_3}^*$, is complex for negative values of δY, therefore, $\delta Y = 0$, is the threshold for stability and has at two potential solutions $\delta = 0$ and $Y = 0$. However, since $\delta \leq 0$, inequality $Y > 0$ determines stability alone i.e.

$$4\alpha^2(3\beta - 2\mu)(\beta + 2\mu) + c^2\beta(7\beta + 16\mu) - 20\alpha c\beta(\beta + 2\mu) > 0. \quad (5.14)$$

Rearranging (5.14) for β yields the following critical threshold for horizontal transfer

$$\beta > {\beta_4}^* = \frac{-4\mu(\alpha-2c)(2\alpha-c)+8\sqrt{(\alpha-c)(2\alpha-c)(2\alpha^2-2\alpha c+c^2)\mu^2}}{(6\alpha-7c)(2\alpha-c)}. \quad (5.15)$$

(ii) The condition at which $\mathcal{E}_3$ has a negative component

The numerator of $n_{ITA_3}^*$ is sufficient for determining the sign of $n_{ITA_3}^*$ as $Z > 0$ given $\alpha > c$. If we let the numerator, $\beta X \pm \sqrt{\delta Y}$, be $-A \pm \sqrt{A^2 - B}$, where $A = -\beta X$ and $B = A^2 - \delta Y$, we can conclude that $n_{ITA_3}^*$ is negative given $A > 0$ and $B > 0$. As A is not globally positive, we must introduce assumptions that limit the inequality to the parameter space of interest. Since $\mathcal{E}_3$ and $\mathcal{E}_1$ are not coexisting stable points, we can assume that, c, is constrained by inequality (2.6). Rearranging inequality (2.6) for c yields

$$\alpha > c > \frac{\alpha\mu(\beta + 4\mu) + \sqrt{\alpha^2\mu^2(16\mu^2 + 8\beta\mu - 7\beta^2)}}{4\mu^2}. \quad (5.16)$$

Using the computer algebra system Mathematica it was determined that within the constrained parameter space of inequality (5.16) A is positive. Thus, $B = 0$ provides a set of critical thresholds for the stability of $\mathcal{E}_3$. Solving $B = 0$ and rearranging for β yields the following two inequalities

$$\beta > \frac{\left(c + \sqrt{(16\alpha - 7c)c}\right)\mu}{2\alpha}, \quad (5.17)$$

$$\beta > \frac{c\mu}{(\alpha - c)}. \quad (5.18)$$

Reassuringly, these critical thresholds are ${\beta_1}^*$ and ${\beta_2}^*$ respectively.

(g) The existence of $\mathcal{E}_4$

The steady-state $\mathcal{E}_4$ has two solutions

$$n_{I_4}^* = 0, \tag{5.19a}$$

$$n_{TA_4}^* = \frac{2(\alpha - c)\left((\beta + \mu) \pm \sqrt{\mu(2\beta + \mu)}\right)}{\beta^2}, \tag{5.19b}$$

$$n_{ITA_4}^* = \frac{(\alpha - c)\left(-\mu(\beta + 2\mu) \pm (\beta - 2\mu)\sqrt{\mu(2\beta + \mu)}\right)}{\beta^2 \mu}. \tag{5.19c}$$

Lets first consider the existence of the additive solution (i.e. $n_{TA_4}^*$ and $n_{ITA_4}^*$ may be simultaneously non-negative). Given our parameter-space is constrained to be positive, it is evident that $n_{TA_4}^* > 0$ given $\alpha > c$. Now assuming $\alpha > c$ we wish to determine if there is a parameter combination in which $n_{ITA_4}^*$ exists. This can be determined by simply reducing the right hand bracket of numerator as follows.

$$-\mu(\beta + 2\mu) + (\beta - 2\mu)\sqrt{\mu(2\beta + \mu)} > 0 \tag{5.20a}$$

$$(\beta - 2\mu)\sqrt{\mu(2\beta + \mu)} > \mu(\beta + 2\mu) \tag{5.20b}$$

$$(\beta - 2\mu)^2 \mu(2\beta + \mu) > \mu^2(\beta + 2\mu)^2 \tag{5.20c}$$

$$(2\beta + \mu)(\beta^2 - 4\beta\mu + 4\mu^2) > \mu(\beta^2 + 4\beta\mu + 4\mu^2) \tag{5.20d}$$

$$2\beta(\beta^2 - 4\beta\mu + 4\mu^2) - 4\beta\mu^2 > 4\beta\mu^2 \tag{5.20e}$$

$$2\beta(\beta^2 - 4\beta\mu) + 4\beta\mu^2 > 4\beta\mu^2 \tag{5.20f}$$

$$2\beta^2(\beta - 4\mu) > 0 \tag{5.20g}$$

This implies $n_{ITA_4}^*$ exists given $\alpha > c$ and $\beta > 4\mu$, reassuring, this is the critical threshold ${\beta_3}^*$. The additive solution is therefore a solution of interest.

Now lets consider the existence of the subtractive solution. Since we know the sign of both solutions changes with $\alpha > c$ we can simply assess when the right hand bracket of $n_{TA_4^*}$ and $n_{ITA_4^*}$ is positive.

Reducing the right hand bracket of the numerator of $n_{TA_4^*}$ yields

$$\beta + \mu - \sqrt{\mu(2\beta + \mu)} > 0 \tag{5.21a}$$

$$(\beta + \mu)^2 - \mu(2\beta + \mu) > 0 \tag{5.21b}$$

$$\beta^2 + 2\beta\mu + \mu^2 \ - \mu(2\beta + \mu) > 0 \tag{5.21c}$$

$$\beta^2 > 0 \tag{5.21d}$$

Therefore, $n_{TA_4^*}$ exists when $\alpha > c$ as β is constrained to be non-negative.

Reducing the right hand bracket of the numerator of $n_{ITA_4^*}$ yields

$$-\mu(\beta + 2\mu) - (\beta - 2\mu)\sqrt{\mu(2\beta + \mu)} > 0, \tag{5.22a}$$

$$-(\beta - 2\mu)\sqrt{\mu(2\beta + \mu)} > \mu(\beta + 2\mu) \tag{5.22b}$$

$$-(\beta - 2\mu)^2\mu(2\beta + \mu) > \mu^2(\beta + 2\mu)^2 \tag{5.22c}$$

$$(\beta - 2\mu)^2\mu(2\beta + \mu) + \mu^2(\beta + 2\mu)^2 < 0 \tag{5.22d}$$

Therefore, $n_{TA_4^*}$ exists when $\alpha < c$ as inequality (5.22) is constrained to be non-positive given $\beta > 0$ and $\mu > 0$.

In conclusion, $n_{TA_4^*}$ exists when $\alpha > c$ while $n_{ITA_4^*}$ exists when $\alpha < c$. Therefore, there is no parameter-space in which the solutions of $n_{TA_4^*}$ and $n_{ITA_4^*}$ are simultaneously non-negative and hence the subtractive solution can be ignored.

(h) Numerical confirmation of analytically derived bifurcations of model (2.5)

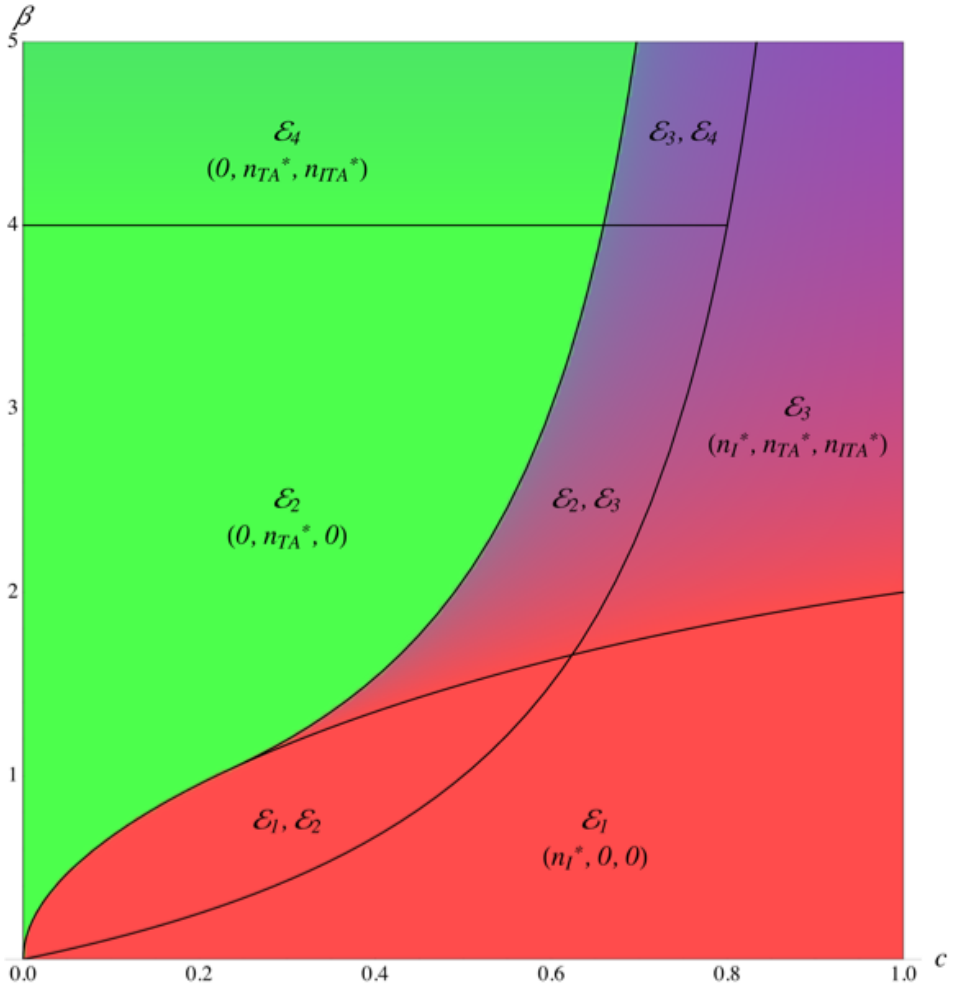

Figure 15. Numerical confirmation of the bifurcations of model (2.5). The proportion of each cell type, I, TA, and ITA, is interpolated by the colors red, green and blue. Each equilibrium outcome was numerically integrated using Mathematica's NDSolve function using initial conditions $n_I(0) = 1, n_{TA}(0) = 10^{-9}$ and parameters $\alpha = 1$ and $\mu = 1$

CHAPTER 4

Addiction extends the existence conditions of bacterial abortive infection

1 INTRODUCTION

Bacteria commonly exhibit the radically altruistic behaviour of *abortive infection*, where a bacterium commits suicide upon infection by an antagonistic virus (bacteriophage or phage). Successful abortive infection prevents, or severely restricts, the maturation of viable phage progeny, but in doing so, renders the cell incapable of further reproduction. Although the reproductive cost associated with the act of suicide is negligible, as cells are moribund and face imminent death, there is typically an appreciable cost associated with the constitutive expression of gene products at all other times. By preventing secondary infections, abortive individuals export an incorporeal public good i.e. a phage-less environment. However, like any public-good, populations of cells that express the costly gene products are susceptible to invasion by non-producer cells that benefit from, but do not contribute to, the reduction of secondary infections, a situation referred to as the "tragedy of the commons" (Hardin, 1968). Recently, the conditions permitting the evolution of abortive infection has received much empirical and theoretical attention. Empirical studies have shown that costly abortive infection determinants may evolve in environments that are structured, where the benefit of abortion preferentially falls upon related individuals (Fukuyo *et al.*, 2012; Berngruber *et al.*, 2013), while in cases where the cost of abortive infection is negligible, little or no genetic structure is required to maintain the altruistic trait (Refardt *et al.*, 2013).

Unhindered lytic bacteriophage infections may generate between 100-300 phage progeny (Birge, 2006), meaning cells that are permissive to infection generate an immense cost to the community in the form of secondary infections. Even the presence of a small proportion of permissive cells could result in population collapse. Furthermore, the cooperative suppression of phage reproduction is complicated by the fact that many phages predate upon multiple bacterial "species". Meaning, in mixed populations composed of unrelated individuals cooperative abortive lineages would be readily undermined by non-cooperative permissive lineages. Thus, the emergence and presence non-cooperative permissive cells is a fundamental problem concerning the evolution of abortive infection and mechanisms that coerce cooperation and dissuade defection would greatly aid in the emergence and persistence of abortive infection.

Abortive infection is typically mediated by toxin-antitoxin complexes that respond to physiological cues associated with phage reproduction, such as the sequestration of amino acids (Aizenman *et al.*, 1996; Hazan & Engelberg-Kulka, 2004). Interestingly, many toxin-antitoxin systems that cause abortive infection have the dual and inseparable phenotype of causing post-segregational killing.

Post-segregational killing or bacterial addiction, is where, upon loss of the gene complex by mutation, recombination or segregation through cell division, the cell is killed by the activity of usually an intra-cellular toxin (Gerdes *et al.*, 1986). When borne by a plasmid, post-segregational killing determinants increase the prevalence of the plasmid in the population by killing cells that fail to inherit the replicon. The first observed post-segregational killing determinant, *hok*/*sok*, was later found to also confer abortive infection against T4 in *E. coli* (Pecota & Wood, 1996), and since then this dual phenotype has been observed of other abortive infection systems (Fineran *et al.*, 2009; Otsuka & Yonesaki, 2012; Dy *et al.*, 2014).

When traits that cause abortive infection also kill genetic miss-segregants, herein referred to as *addictive abortive infection*, killing is directed exclusively towards individuals that no longer bear the altruistic suicide trait. That is, non-cooperative cheats are actively punished (by being killed) when they emerge. Thus, directed punishment by post-segregational killing may act to maintain the cooperative allele at high prevalence. Strong reciprocity, the altruistic punishment of non-cooperators, is thought to promote cooperative behaviours in human groups (Boyd *et al.*, 2003; 2010). However, in microbiological communities cooperative punishers are susceptible to invasion by cooperative non-punishers unless the two phenotypes, punishment and cooperation are perfectly linked (Lehmann *et al.*, 2007; Inglis *et al.*, 2014).

We propose that the perfectly linked dual phenotype of many abortive infection determinants – post-segregational killing – extends the existence conditions of abortive infection by punishing non-cooperative cheats that emerge by either mutation or loss of the locus. We develop a mathematical model to examine the population

biology of plasmid-borne addictive abortive infection determinants. Specifically, we examine how conjugative plasmids, which are hypothesized to maintain cooperative bacterial genes (Smith, 2001; Rankin *et al.*, 2011), and post-segregational killing affect the stability of abortive infection. We find that the effect of addiction on the prevalence of the abortive infection is most pronounced when rates of segregational loss are high, like those found in mixed plasmid populations, or when horizontal transfer is restricted, such as low cell density environments. Moreover, we predict that plasmid-borne addiction provides a novel mechanism to ensure that the cooperative trait is maintained in the presences of plasmid defectors (i.e. plasmids that have lost the abortive infection gene) or cognate replicons that are competing for vertical reproduction. Finally, we discuss the relevance of our work with respect to previous models and experiments of strong reciprocity in bacterial systems.

2 MODEL & RESULTS

(a) Permissive-only model

We start with a simple bacteria-virus model that follows the densities of bacteria that permit normal viral reproduction (permissive), P, and an obligate lytic bacteriophage (bacteria virus), V. We assume bacteria grow in a resource-constrained environment and that they are limited by the carrying capacity of the environment, k, modeled using the standard logistic law. The infection of permissive bacteria, P, by phage particles, V, occurs at rate, ϕ, assuming mass-action kinetics. Upon infection and successful subversion of host defences, phage reproduction quickly ensues culminating in lysis of the host and release of phage progeny. For simplicity, we assume infection, reproduction and release of phage particles occurs instantaneously. For every phage infection b viral progeny are produced (burst-size) whilst the focal infecting phage is removed, yielding a total gain of $b-1$ phage per infection. These assumptions yield the following system of ordinary differential equations,

$$\frac{dP}{dt} = \overbrace{P\left(1-\frac{P}{k}\right)}^{birth\ \&\ death} - \overbrace{\phi PV}^{infection}, \tag{2.1a}$$

$$\frac{dV}{dt} = \overbrace{\phi VP(b-1)}^{infection\ and\ death}. \tag{2.1b}$$

In the absence of phage, permissive bacteria exist at the exterior equilibrium $E:(P,V)=(k,0)$. Phage may invade if the per capita rate of change of V at equilibrium E is greater than zero, $\frac{dV}{dt}\frac{1}{V}>0$. Therefore, as ϕ and k are strictly positive, phage invade when their burst-size overcomes loss due to infection, $b>1$. This invasion threshold may also be expressed as a reproductive ratio where $R_0 = b/1$.

(b) Permissive-Abortive model

We now introduce a conjugative plasmid bearing an abortive infection locus (abi^+) that promotes cell death upon phage infection, with density being A and total bacterial density being $N=P+A$. We assume that the transfer of plasmids to permissive cells

(abi^-), P, from abortive cells (abi^+), A, occurs at rate β assuming mass action kinetics (Levin *et al.*, 1979). The *abi* locus is lost through miss-segregation of the plasmid at rate s. Plasmid segregation typically occurs at extremely low frequencies in clonal plasmid populations. However, in mixed populations the frequency of plasmid loss per generation may increase by 5-6 orders of magnitude due to co-infection and reproductive incompatibility (Condit & Levin, 1990). Although we do not explicitly account for co-infection in our model, it is implicitly parameterised by the birth-independent segregation process s (we explore the relaxation of this assumption in the discussion). Over-expression of the toxin in some abortive infection determinants, such as *toxIN,* results in reversible cell arrest (i.e. bacteriostasis), whilst in other systems, such as *hok/sok*, toxin activity causes irreversible cell death.[5] We will model the later case where toxin activity causes post-segregational killing, specifically, most plasmid-free miss-segregants die but a proportion, ε, survive miss-segregation and become permissive to viral infection. The plasmid confers a cost, c, upon the host. This cost includes the effects of the *abi* locus. The infection of permissive and abortive cells, P and A, by virus, V, occurs at rate, ϕ, and is once again modelled using mass action kinetics. Permissive bacteria allow the virus to reproduce unhindered, permitting a maximal virus burst-size, b, whereas abortive bacteria limit the number of virus progeny to a proportion of the maximal burst-size, $0 < \omega < 1$. As before, each infection removes the focal infecting phage from the population. These assumptions yield the following system of ordinary differential equations.

$$\frac{dP}{dt} = \overbrace{P\left(1-\frac{N}{k}\right)}^{birth\ \&\ death} - \overbrace{\beta PA}^{transfer} + \overbrace{\varepsilon sA}^{PSK\ failure} - \overbrace{\phi PV}^{infection} \tag{2.2a}$$

$$\frac{dA}{dt} = \overbrace{A\left(1-\frac{N}{k}\right)}^{birth\ \&\ death} + \overbrace{\beta AP}^{transfer} - \overbrace{sA}^{segregation} - \overbrace{cA}^{cost} - \overbrace{\phi AV}^{infection} \tag{2.2b}$$

$$\frac{dV}{dt} = \overbrace{\phi VP(b-1)}^{infection\ of\ P} + \overbrace{\phi VA(\omega b-1)}^{infection\ of\ A} \tag{2.2c}$$

[5] Despite *toxIN* being bacteriostatic it still stabilizes plasmid inheritance (Fineran *et al.*, 2009) and thus could also act to repress the emergence of permissive cells.

Let's begin by examining the dynamics of permissive and abortive cells in the absence of viruses. As the *abi* locus is plasmid-borne, the dynamics of permissive and abortive cells are similar to that derived in Chapters 2 & 3. Bacteria are logistically constrained by the carrying capacity of the of the environment and therefore in the absence of *abi*-plasmids persist at equilibrium $E_1: (P, A, V) = (k, 0, 0)$. The stability of E_1 is lost when the rate of plasmid transfer at equilibrium, βk, outweighs the cost of plasmid carriage and segregational loss, $\beta k > c + s$. So long as this invasion condition is met, the plasmid-carrying population will increase until the two populations arrive at the bacterial coexistence equilibrium, $E_2: (P, A, V) = ({P_2}^*, {A_2}^*, 0)$[6], with permissive cells persisting due to their fecundity advantage and continuous generation from plasmid-carrying cells via segregational loss and failure of post-segregational killing.

[6] An analytical expression for E_2 was computed using Mathematica, however, it has been omitted due to its size.

Variables & parameters	Description
$\boldsymbol{P}$	Density of hosts that are permissive to phage reproduction
$\boldsymbol{A}$	Density of hosts that undergo cell apoptosis (abortion) upon phage infection thereby limiting the burst-size
$\boldsymbol{N}$	Total density of bacterial hosts (i.e. $N = P + A$)
$\boldsymbol{V}$	Density of phage (bacterial viruses)
$\boldsymbol{k}$	Carrying capacity of the bacterial population
$\boldsymbol{\phi}$	Phage infectivity rate constant
$\boldsymbol{b}$	Phage burst-size
$\boldsymbol{\omega}$	Proportion of phage progeny that survive abortion (efficiency of abortion)
$\boldsymbol{\beta}$	Plasmid conjugation rate constant
$\boldsymbol{c}$	Plasmid cost (including the *abi* locus)
$\boldsymbol{s}$	Rate of plasmid segregation
$\boldsymbol{\varepsilon}$	Proportion of plasmid miss-segregants that survive post-segregational killing (efficiency of addiction).

Table 2. Summary of variables and parameters.

(i) Factors influencing the density of permissive cells

Crucial to the success of invading phage is the presence of permissive cells, without which infections would fail to produce sufficient viable progeny. Let Q^* be the proportion of permissive cells at equilibrium, $Q^* = P^*/(P^* + A^*)$. We now examine how the density of permissive and abortive cells at equilibrium E_2 changes according to segregational loss, the efficiency of post-segregational killing, and horizontal transfer of the *abi* locus.

Addiction relaxes the need for highly faithful plasmid inheritance (Figure 16, top). As the rate of segregational loss, s, increases towards the threshold of plasmid invasion, $s \to \beta k - c$, efficacious addiction (i.e. decreasing ε) becomes crucial for suppressing the emergence of permissive cells. Plasmid inheritance machinery such as copy-control and partitioning loci ensure low levels of stochastic miss-assortment of cognate plasmid replicons at cell division. However, in mixed-plasmid populations these mechanisms rapidly force incompatible plasmids into distinct lineages (Condit & Levin, 1990). This is of particular importance when considering the generation of mutants lacking the *abi* locus at the co-existence equilibrium E_2. Mutant *abi*$^-$ plasmids arising at E_2 would rapidly segregate from cooperative plasmids. However, if the cooperative locus had a dual function of abortive infection and post-segregational killing, the generation of permissive mutants at E_2 would be severely restricted.

Analogously, addiction relaxes the need for high rates of horizontal transfer of the cooperative locus (Figure 16, bottom). As the rate of horizontal transfer decreases towards the critical threshold, $\beta \to (s + c)/k$, addiction becomes increasingly important for suppressing the emergence of permissive individuals. Thus, addiction would aid in the maintenance of cooperative loci under conditions that limit horizontal transfer.

At higher levels of horizontal transfer, the influence of addiction becomes less important. Permissive cells arising from segregational loss would be quickly re-colonised by horizontal transfer. However, horizontal transfer alone may not be

sufficient for maintaining plasmids in mixed-populations; incompatible uncooperative *abi*$^{-}$ plasmids of co-infected cells would compete for vertical transmission and compromise the ability of the cooperative *abi*$^{+}$ plasmid to be fixed in descendent cells. Addiction may therefore also provide a benefit by eliminating plasmids that compete for vertical reproduction. Although our model does not explicitly account for competition between competing plasmids, we elaborate further in the discussion on the likely utility of addiction complexes for maintaining relatedness in mixed-plasmid populations.

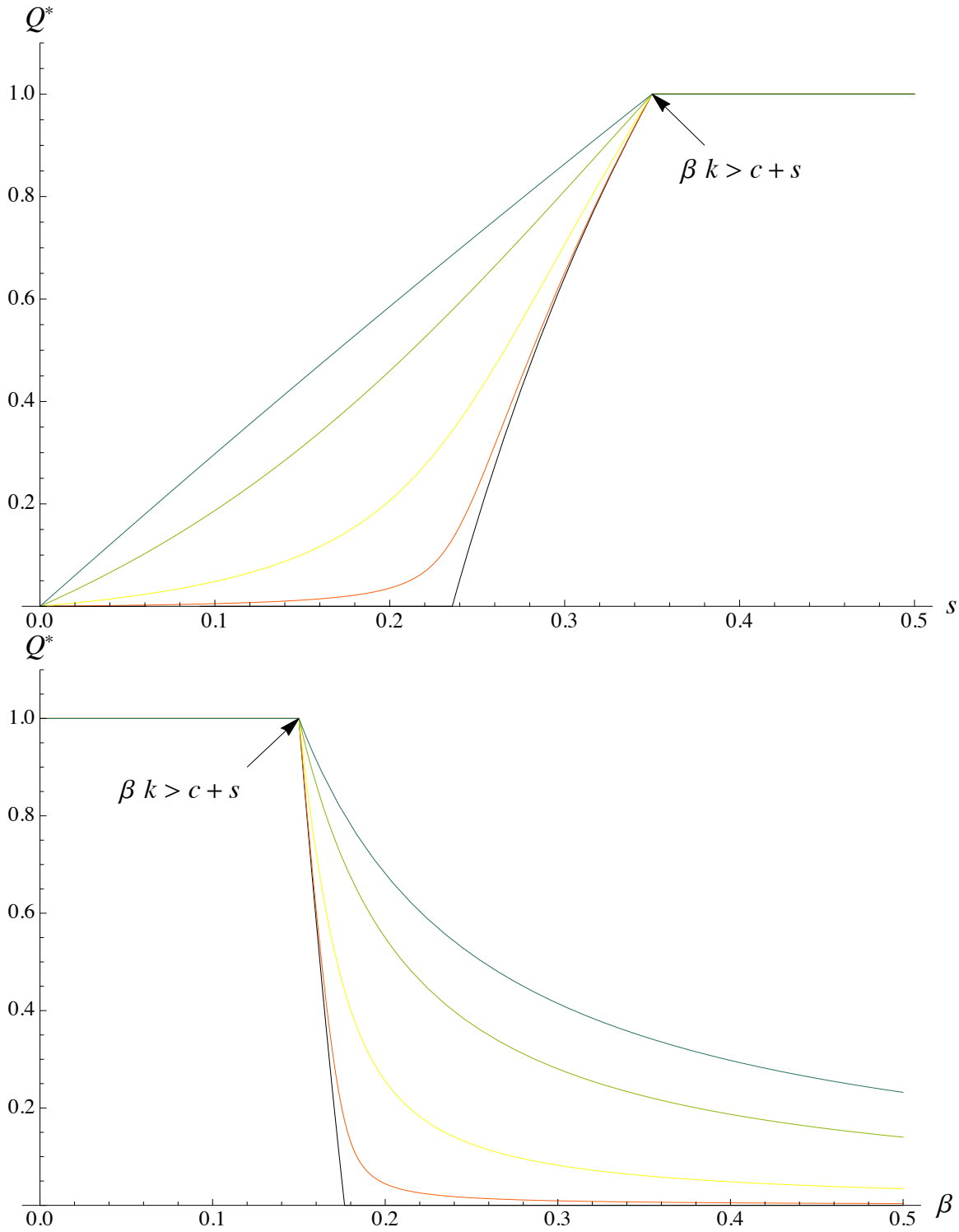

Figure 16. (top): The proportion of permissive individuals at equilibrium, Q^*, as a function of segregational loss, s, of the *abi* locus. The effect of efficacy of addiction, ε, on the emergence of permissive individuals is shown for $\varepsilon = (0,\ 10^{-2},\ 10^{-1}, 0.5, 1)$ in black, orange, yellow, green and blue respectively. Remaining parameters were $k = 1$, $c = 0.05$ and $\beta = 0.4$. (bottom): Q^* as a function of horizontal transmission, β. Parameters are identical to *top* with the rate of segregational loss being $s = 0.1$.

(ii) Phage invasion - bacterial population collapse

Provided the threshold for invasion of cooperative plasmids is met, i.e. $\beta k > c + s$, permissive and abortive cells coexist at equilibrium E_2 where the proportion of permissive cells varies according to the rate of segregational loss, cost, horizontal transfer of the cooperative plasmid and the efficiency of post-segregational killing. However, if the density of permissive cells is sufficiently large, then phages invade. Invasion results in the collapse of both permissive and abortive populations. The intercept of E_2 and the phage-bacterial co-existence equilibrium, $E_3: (P, A, V) = ({P_3}^*, {A_3}^*, {V_3}^*)$[7], represents this tipping point. Specifically, the intercept of E_2 and E_3 is a transcritical bifurcation which occurs when ${V_3}^* = 0$. As E_3 is an unstable node, bacteria and phage never stably co-exist, instead the system converges upon the phage-only equilibrium $E_4: (P, A, V) = (0, 0, {V_4}^*)$[8]. Thus, the proportion of permissive cells at E_3, ${Q_3}^*$, represents the maximum proportion of permissive cells allowed before E_2 loses stability, $\tilde{Q}$ i.e. $\tilde{Q} = {Q_3}^* = {P_3}^*/({P_3}^* + {A_3}^*)$. Any greater proportion of permissive cells results in the collapse of both bacterial genotypes. As such, E_3 can be used to derive analytical expressions relating to the conditions required for viral invasion.

Phage may invade from rare when ${V_3}^* > 0$. Therefore, the non-trivial condition (i.e. when $\omega b < 1$) for phage invasion is (see Appendix 5(b) for derivation)

$$kb\beta(1 - c - s)(\omega b - 1)(\omega - 1) + \big((c + s)(\omega b - 1) - s\epsilon(b - 1)\big)\big(k\beta(\omega b - 1) - b(\omega - 1)\big) > 0. \quad (2.3)$$

With inequality (2.3) we can examine the relationship between plasmid segregational loss, s, and efficiency of post-segregational killing, ε. The critical threshold b^*[9] is the maximally tolerated burst-size that does not lead to systemic growth of the phage. The required burst-size for invasion, b^*, increases with the suppression of permissive cells. For high rates of plasmid loss, like those encountered in mixed-incompatible plasmid populations, increases in the efficiency of post-segregational killing

[7] For an analytical expression of E_3 see Appendix (a)
[8] Analytical expression not shown
[9] Due to the size of the expression it has been omitted from this text.

dramatically increases the required burst-size, b^* (Figure 17). However, this effect diminishes as the rate of plasmid segregation decreases towards rates that are encountered in clonal-plasmid populations.

Phage invasion success depends on the proportion of permissive cells at equilibrium, Q^*, the threshold of which, $\tilde{Q}$, is a function of two phage-specific parameters; burst-size, b, and the efficiency of reproducing within abortive cells, ω. It can be shown (see Appendix 5(c) and 5(d) for derivation) that the proportion of permissive cells at the phage invasion threshold is

$$Q^* > \tilde{Q} = {Q_3}^* = \frac{1 - \omega b}{b - \omega b}, \tag{2.4}$$

which can be restated as an R_0

$$R_0 = Q^* b + (1 - Q^*) w b = \frac{P^* b + A^* w b}{P^* + A^*}, \tag{2.5}$$

where the terms $Q^* b$ and $(1 - Q^*) w b$ are the per capita phage progeny produced by the infection of permissive and abortive cells respectively. When their sum is greater than 1, i.e. $R_0 > 1$, phage will invade.

Inequalities (2.3), (2.4) and (2.5) are equivalent expressions of the threshold for phage invasion. From inequality (2.4) we can identify two restrictive conditions for the evolution of abortive infection (Figure 18): 1. When $\omega > b^{-1}$, viruses may invade bacterial populations consisting solely of abortive cells, i.e. $\lim_{\omega \to b^{-1}} \tilde{Q} = 0$. In this situation, every infection of an "abortive" cell yields a single phage progeny thereby permitting phage survival on "abortive" cells alone. A typical burst-size of 200 would therefore require abortion to be at least 99.5% effective at preventing the maturation of phage particles. 2. Assuming effective abortive infection, $\omega \to 0$, the critical threshold of permissive cells is approximated by $\tilde{Q} = b^{-1}$, i.e. $\lim_{\omega \to 0} \tilde{Q} = b^{-1}$. Again, for a typical burst-size of 200, the proportion of uncooperative permissive cells may not exceed 0.05 if the population of bacteria was to avoid collapse. Together, these limits imply that $\tilde{Q}$ exists within the bounds $0 < \tilde{Q} \leq b^{-1}$, where the

lower and upper bounds are constrained by the limits $\omega \to b^{-1}$ and $\omega \to 0$, respectively.

Both of these conditions assume that the macroscopic behaviour of the population level model (mean-field) is an accurate approximation of the microscopic behaviour at the individual level. However, the probability of a rare virus particle encountering a rare permissive individual will be heavily influenced by chance, and thus, stochastic effects may render the mean-field model inaccurate in certain situations. In the following section we develop a stochastic individual-level model of system (2.2) and investigate the microscopic behaviour specifically with respect to the maximally tolerated proportion of permissive cells, $\tilde{Q}$.

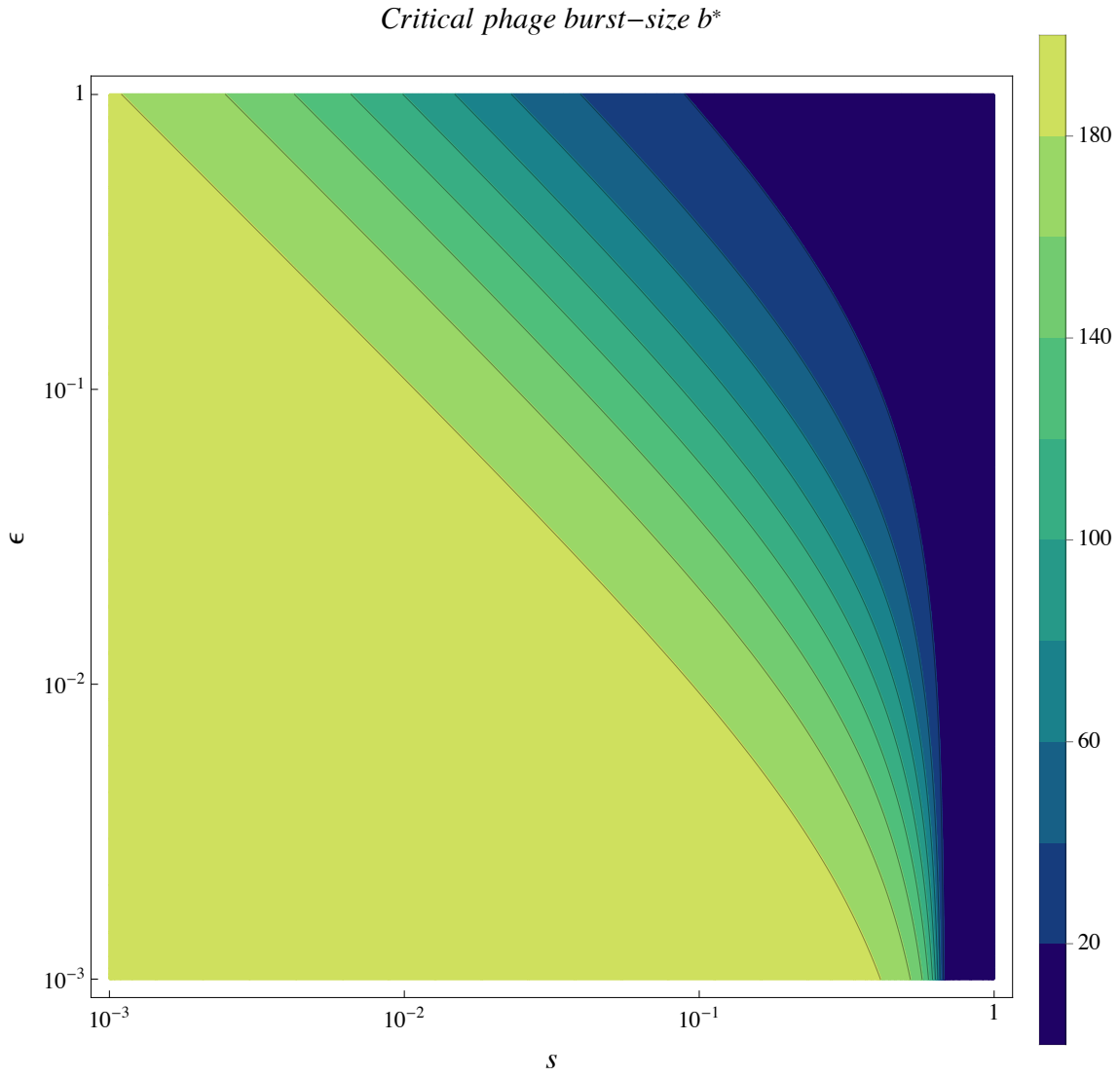

Figure 17. Critical phage burst-size b^* as a function of efficacy of addiction, ε, and rate of segregational loss, s. As the rate of segregational loss increases highly efficacious addiction, $\varepsilon \to 0$, dramatically increases the required burst-size of the invading phage. Whereas, when segregation loss is negligible, highly efficacious addiction has little influence on the critical phage burst-size. Remaining parameters were $k = 10^6, \beta = 2/k, c = 0.01, \phi = 2/k$ and $\omega = 0.05$.

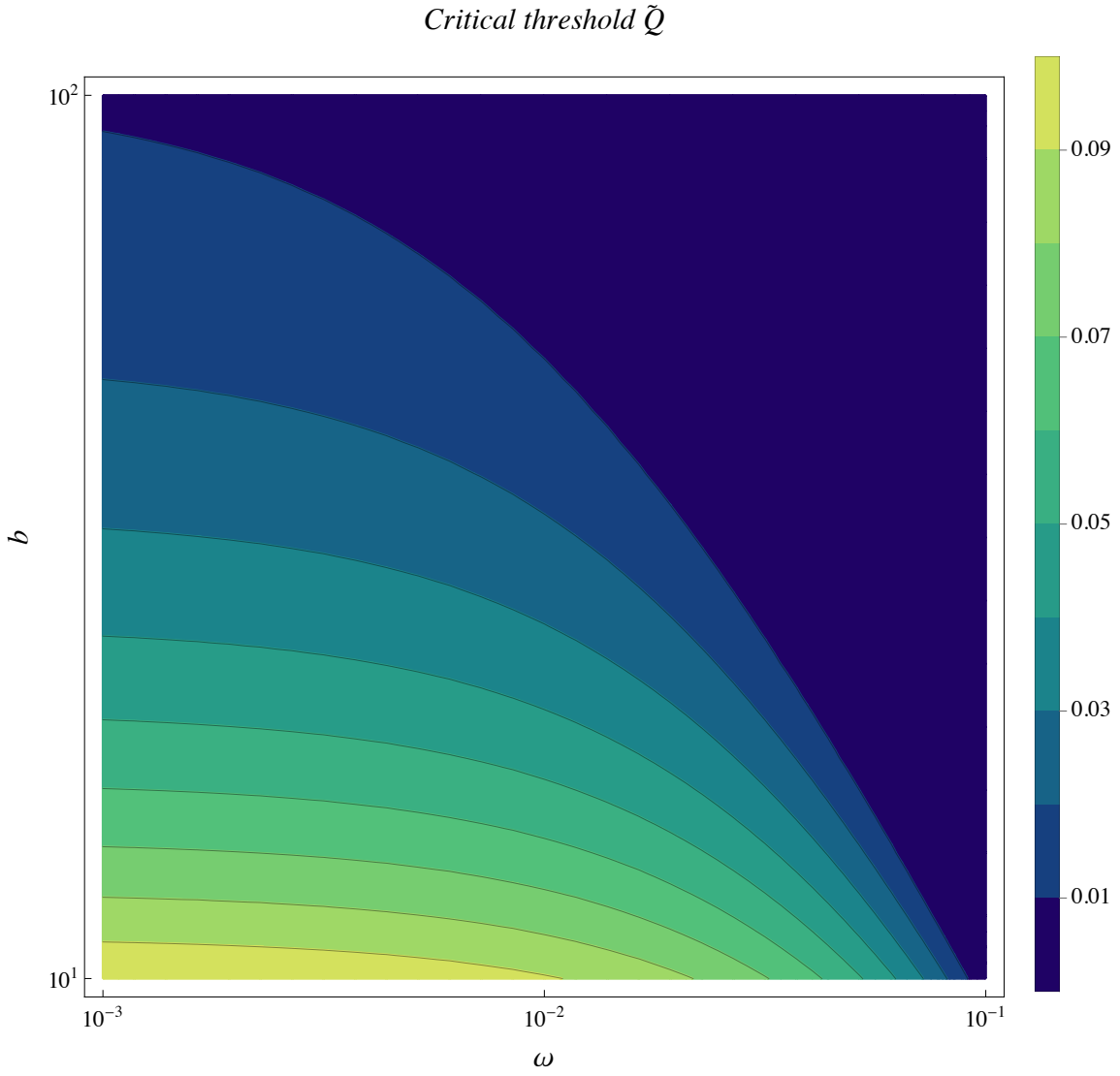

Figure 18. The maximally tolerated proportion of permissive cells, $\tilde{Q}$, by burst-size, b, and efficiency of abortion, ω. The critical threshold, $\tilde{Q}$, is the maximally tolerated proportion of permissive cells above which the bacteria population will collapse due to systemic phage infection. When the efficiency of abortion is high, $\omega \to 0$, $\tilde{Q}$ can be approximated by b^{-1}. Whereas, as the efficiency of abortion is relaxed, $\omega \to b^{-1}$, populations consisting of only "abortive" cells, $\tilde{Q} = 0$, permit systemic phage infection.

(c) Individual-level model

Using the macroscopic or mean-field behaviour of model (2.2) we have shown that phage invasion depends on the proportion of permissive cells, Q^*, being greater than the critical threshold, $\tilde{Q}$. However, when the proportion of permissive cells is small, $Q^* \ll 1$, invasion relies on a rare virus encountering a relatively rare permissive cell. Therefore, the threshold for invasion is likely to be far more restrictive when stochastic effects are accounted for. We now develop an individual-level model to examine the microscopic behaviour of model (2.2) with respect to phage invasion.

Let p denote the number of permissive cells, a the number of abortive cells, v the number of phage and n the total number of bacterial cells, i.e. $n = p + a$. Then $T(p', a', v'|p, a, v)$ is the probability of transitioning from state (p, a, v) to state (p', a', v'). The following set of probabilistic transitions is an individual-level model of the mean field population-level model (2.2)

$$T(p+1, a, v|p, a, v) = p, \tag{2.6a}$$

$$T(p-1, a, v|p, a, v) = p\frac{n}{k}, \tag{2.6b}$$

$$T(p, a+1, v|p, a, v) = a, \tag{2.6c}$$

$$T(p, a-1, v|p, a, v) = a\left((1-\varepsilon)s + c + \frac{n}{k}\right), \tag{2.6d}$$

$$T(p-1, a+1, v|p, a, v) = \beta pa, \tag{2.6e}$$

$$T(p+1, a-1, v|p, a, v) = \varepsilon sa, \tag{2.6f}$$

$$T(p-1, a, v+b-1|p, a, v) = \phi pv, \tag{2.6g}$$

$$T(p, a-1, v+\omega b-1|p, a, v) = \phi av. \tag{2.6h}$$

Model (2.6) constitutes an individual-level representation of the population-level model, (2.2). To check the validity of our model, we first test that the average (or

mean-field) behaviour of the individual-level model is identical to that of the population-level model. Using the R package GillespieSSA[10], we generated 10,000 stochastic realisations of model (2.6) using Gillespie's direct method (Gillespie, 1977). Since we predict stochastic effects to occur when Q^* and $v(0)$ are small, we evaluated the parameter-space $\omega = 0$, $b = 10$, $Q = 0.5$ and $v(0) = 100$ to ensure stochastic effects are unlikely to be captured during validation. In this parameter-space we found perfect agreement between the average behaviour of the individual-level model and the population-level model (see Appendix Figure A1).[11]

When integrated, population-level models yield a single solution and deterministically converge upon a single fixed point (e.g. bacteria become extinct or phage become extinct). However, integration of individual-level models yield different solutions for each realisation, including possibly tending towards different fixed points[12] (e.g. sometimes bacteria become extinct and sometimes phage become extinct). Stochastic integration of model (2.6) shows that when phages are initially rare and Q^* is close to $\tilde{Q}$ invasion is no longer deterministic but instead probabilistic, where equilibria E_2 and E_4 are bi-stable (Figure 19).

We can further understand the time evolution of model (2.6) using probability mass functions. Let n be the total number of bacteria, $N(t)$ be the size of the population at time t (in a single realisation), and $P_n(t)$ be the probability that n bacteria exist at time t, $P_n(t) = Pr(N(t) = n)$. Here, $P_n(t)$ is the probability mass function of bacterial cells at time t and can be computed numerically using repeated stochastic integration (Figure 20).

Stochastic effects at the microscopic level result in phage invasion being a probabilistic event. We now examine in greater detail how the prevalence of permissive cells influences the probability of phage invasion.

[10] http://cran.r-project.org/web/packages/GillespieSSA/
[11] Due to the number of transitions it was simply too difficult to recover the corresponding mean-field model using van Kampen's large-N expansion of the master equation (Kampen, 2007).
[12] Here "fixed point" is the ensemble average of enough realisations that the point can be considered (statistically) stationary and may correspond to a fixed point in the population-level model

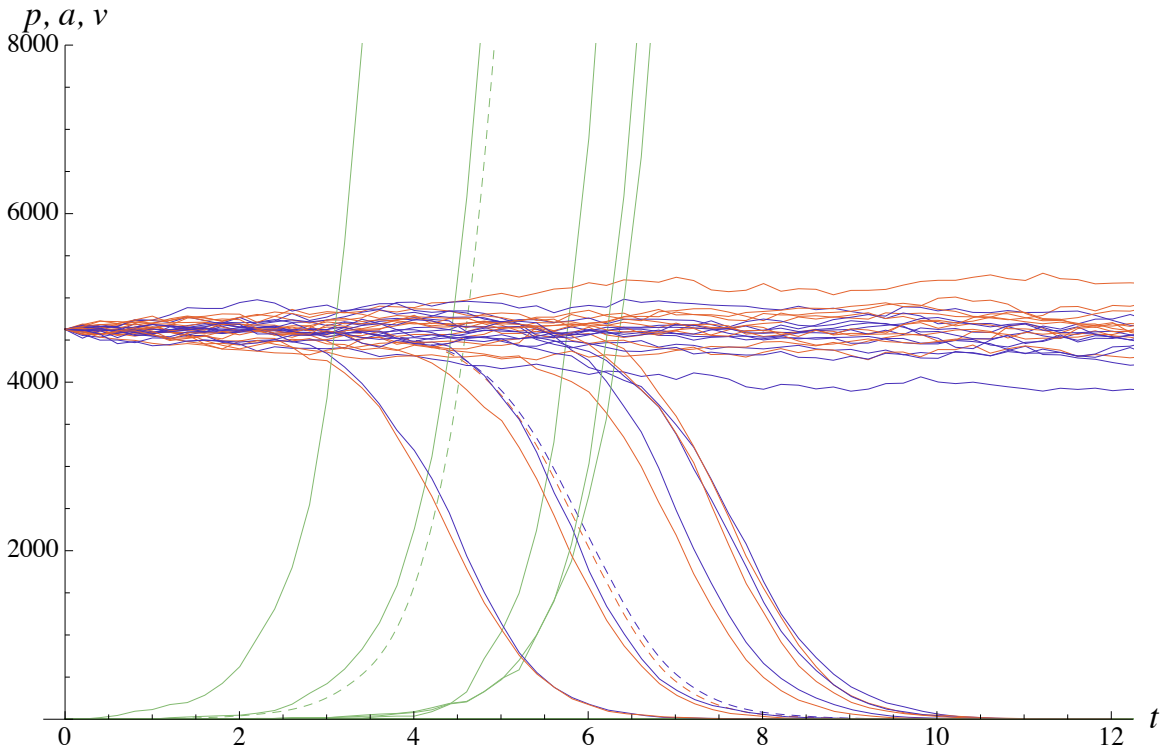

Figure 19. Stochastic integration of model (2.6) using Gillespie's direct method. Permissive cells, abortive cells and phage are in blue, orange and green respectively. Thin lines represent stochastic realisations (totalling 16) while dashed lines are the mean-field result from the population level model. Here we see that phage invasion or bacterial extinction is reliant on stochastic effects where simulations using identical conditions may converge upon different equilibria (i.e. bi-stability). Parameters were $k = 10^4$, $c = 0.1$, $s = 0.05$, $\beta = 1.62659 \times 10^{-5}$, $\phi = 5 \times 10^{-5}$, $\omega = 0$, $b = 10$ (i.e. $Q^* = 0.5$ and $\tilde{Q} = 0.1$). Initial conditions were $p(0) = 4626$, $a(0) = 4626$ and $v(0) = 100$.

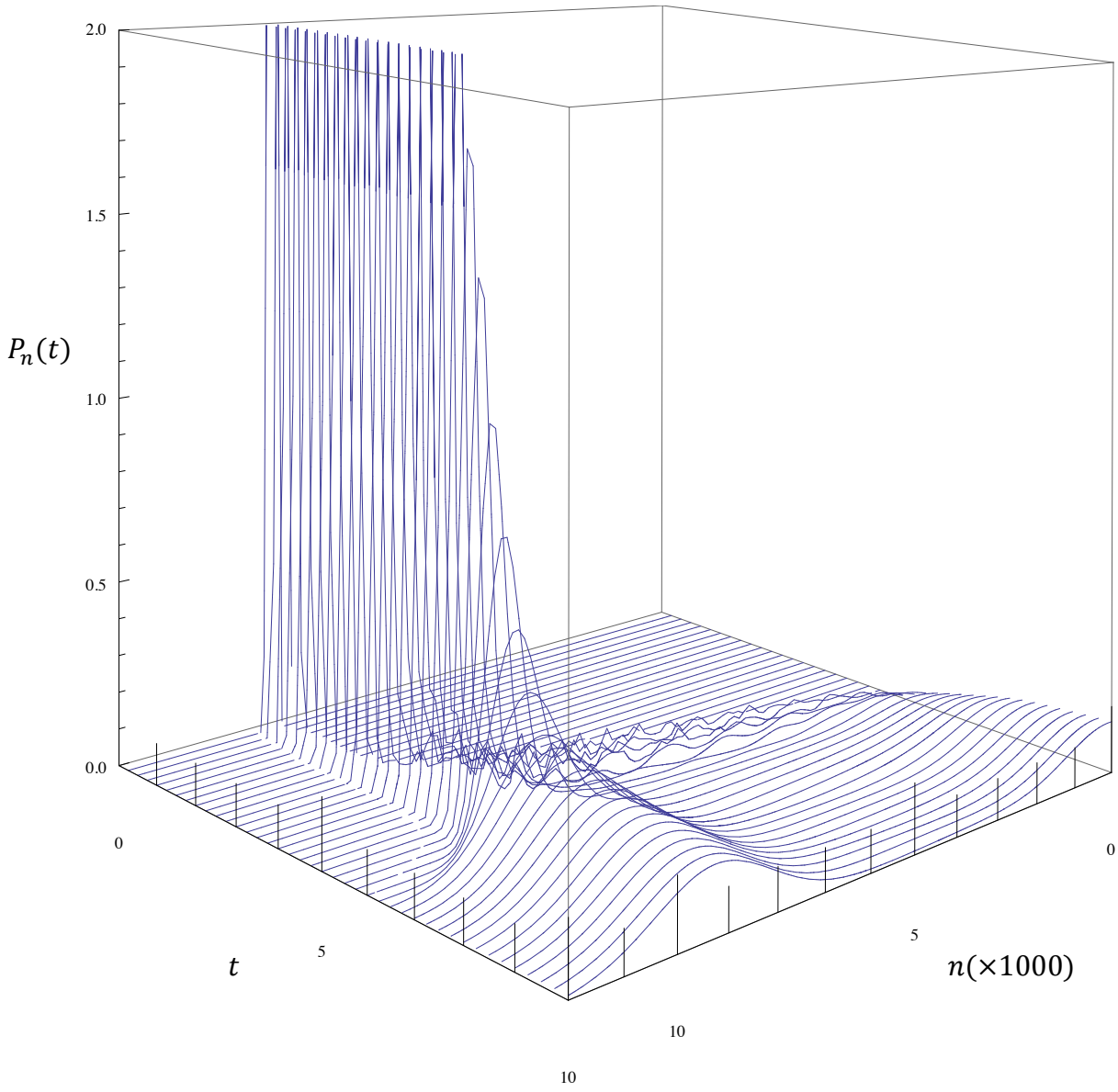

Figure 20 Probability mass function of n cells existing at time t i.e. $P(N(t) = n)$ upon integrating model (2.6). Here, like in Figure 19, we see that invasion is probabilistic where some realisations result in the collapse of the bacterial population while in others the phage is cleared (i.e. bi-stability). The continuous probability mass function was derived numerically using 500 discrete stochastic realisations and Mathematica's 9 SmoothKernelDistribution function. Parameters were same as in Figure 19.

(ii) Probability of invasion

To examine stochastic effects relating to phage invasion we considered three critical invasion thresholds, $\tilde{Q} = (0.5, 0.2, 0.1)$, with burst-sizes, $b = (2, 5, 10)$, and efficiency of abortion, $\omega = 0$.[13] For each critical invasion threshold we computed the probability of invasion for the following proportions of permissive cells, $Q^* = (0.05, 0.1, 0.15 \ldots 1)$. In our model, phages have no intrinsic death rate, persist indefinitely as extracellular particles, and are removed by the infection only (successful or otherwise). Thus, when a population is perturbed there is no indefinite co-existence, either the bacterial population or the phage population is eliminated. As such, we define the probability of invasion as the probability that all bacterial cells are eliminated when perturbed by a phage $\overline{I} = P(N(t_{max}) = 0 \mid v(0) = 1)$. Where t_{max} is a time period sufficiently large to allow either the total collapse of the bacterial population or extinction of phages.

Earlier we showed that the proportion of permissive individuals, the multivariate function Q^*, is independent of the threshold of invasion $\tilde{Q}$. Specifically, we showed that phage invade when,

$$Q^*(k, \beta, c, s, \epsilon) > \tilde{Q}(\omega, b) = \frac{1 - \omega b}{b - \omega b}. \qquad (2.7)$$

The independence of Q^* and $\tilde{Q}$ simplifies our analysis of stochastic effects. Instead of examining the effect of each parameter on the probability of invasion, we can simply vary Q^* using a single arbitrary parameter. The parameter-space used in our simulations, specifically values for Q^*, are summarized in Table A2 and Figure A2.

For each parameter combination we simulated 50,000 stochastic realizations using Gillespie's direct method (Gillespie, 1977)[14]. The initial population size of abortive and permissive individuals, a and p, corresponded to a discrete realization of equilibrium E_2 for each Q^* (parameters described in Table A2). Each population was exposed to a single phage, $v(0) = 1$, and integrated using Gillespie's direct method

[13] Unfortunately, simulations using larger burst-sizes were too computationally intensive, however, as will be shown, simulations in this parameter space can be approximated by a very simple analytical expression which is most likely also accurate for larger burst-sizes.
[14] We used the R library GillespieSSA. It provides an easy to use implementation of Gillespie's direct method.

until either the bacterial population or phage population went extinct. The probability of invasion was then calculated by determining the proportion of simulations where phage invaded (Table 3 and Figure 21).

(iii) Analytical approximation for extinction and invasion

In the restricted parameter-space where abortion efficiency is absolute, $\omega = 0$, infection of a permissive cell results in b phage progeny, with probability Q^*, whereas the infection of an abortive cell results in zero progeny, with probability $1 - Q^*$, and in both cases, infection destroys the focal infecting phage. Let the probability that a phage during its lifetime (i.e. a single infection) produces 0 or b progeny be p_0 and p_b respectively, with $p_0 = 1 - Q^*$ and $p_b = Q^*$. Here, Q^*, can be thought of as the ensemble average of the starting proportion of permissive cells in the bacterial population. The probability of phage extinction, ϵ, at generation n is a simple Galton-Watson process and can be expressed using the recurrence relation

$$\epsilon_n = p_0 + p_b {\epsilon_{n-1}}^b .. \quad (2.8)$$

Although a Galton-Watson process models discrete generations, and thus does not capture the time evolution dynamics of our model, it remains a qualitatively accurate predictor of the probability of ultimate extinction. With increasing generations the probability of extinction converges upon a probability of ultimate extinction, $\overline{\epsilon}$, which occurs when $\epsilon_n = \epsilon_{n-1}$, therefore,

$$\overline{\epsilon} = p_0 + p_b \overline{\epsilon}^b . \quad (2.9)$$

The probability of invasion, $\overline{I}$, is then simply $\overline{I} = 1 - \overline{\epsilon}$. Equation (2.9) is in close agreement with numerically derived probabilities for invasion of a rare phage i.e. $v = 1$ (Figure 21).

Together, these numerical and analytical results show that phage invasion from rare is probabilistically unlikely when the proportion of permissive cells, Q^*, is at or slightly above the threshold of permissive cells, $\tilde{Q}$. This effect is owing to either the rarity of permissive cells or the initially small number of invading phage. In general, this means the pressure to maintain permissive cells as extreme minority populations is relaxed due to stochastic effects when phage invade from rare.

Q^*	$\bar{I}$		
	$b = 10$	$b = 5$	$b = 2$
0.05	0	0	0
0.1	0.00866	0	0
0.15	0.09	0.0003	0
0.2	0.165	0.015	0
0.25	0.233	0.0987	0
0.3	0.291	0.203	0
0.35	0.347	0.286	0
0.4	0.399	0.354	0.0001
0.45	0.452	0.42	0.0024
0.5	0.503	0.486	0.0345
0.55	0.553	0.54	0.157
0.6	0.598	0.593	0.32
0.65	0.65	0.648	0.463
0.7	0.702	0.705	0.573
0.75	0.75	0.753	0.667
0.8	0.801	0.799	0.756
0.85	0.85	0.85	0.824
0.9	0.9	0.9	0.889
0.95	0.95	0.95	0.947
1	1	1	1

Table 3. Probability of invasion, $\bar{I}$, for varying proportions or permissive cells, Q^*, and burst-sizes $b = (2, 5, 10)$ (with efficiency of abortion $\omega = 0$, and remaining parameters being the same as in Table A2). Each probability was derived from 50,000 stochastic realisations using an initial phage perturbation of $v(0) = 1$.

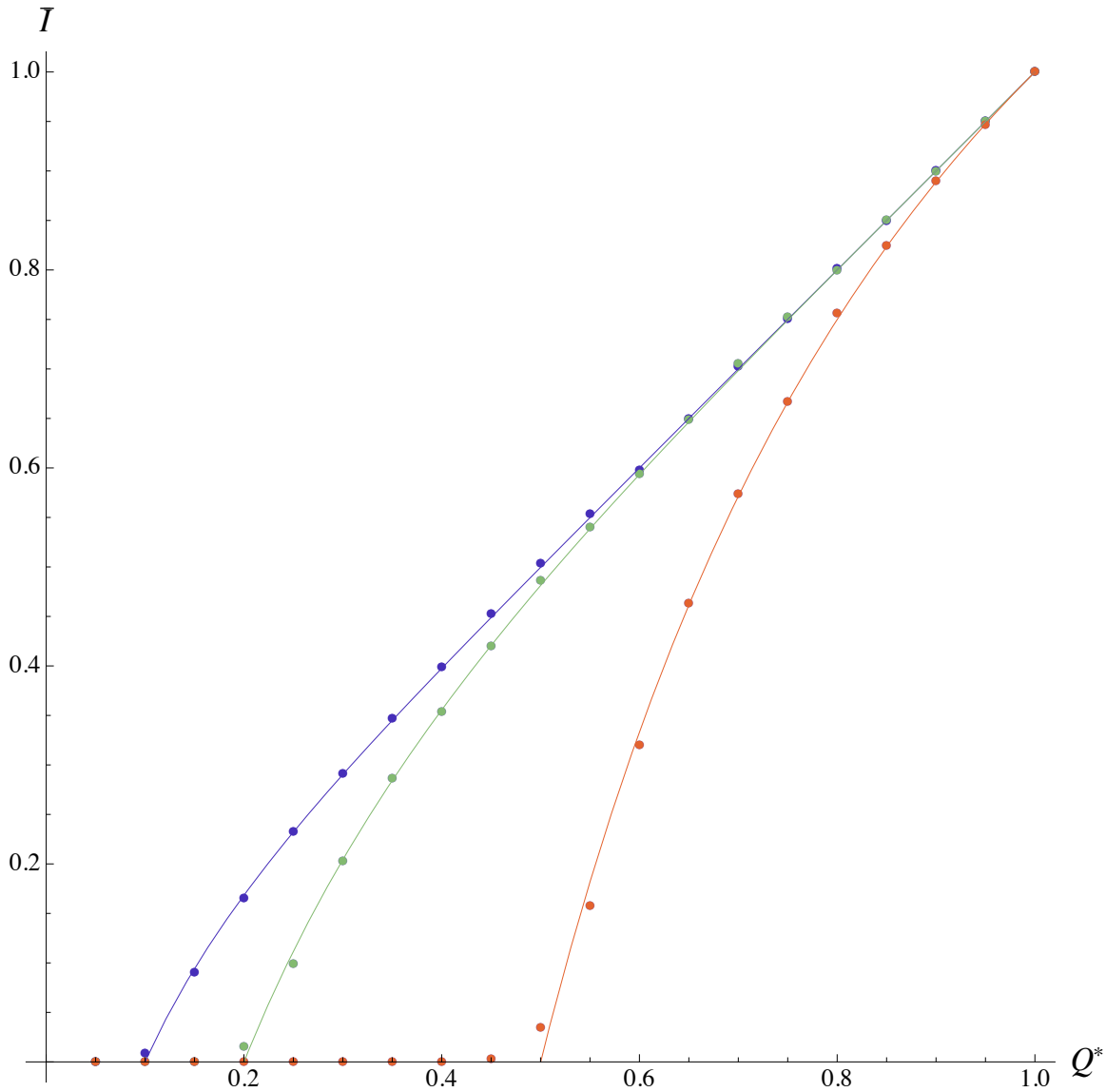

Figure 21. Probability of invasion, $\bar{I}$ or $P_n(t_{max}) = 0$, as a function of permissive cells at equilibrium, Q^*. We consider three critical invasion thresholds, $\tilde{Q} = (0.5, 0.2, 0.1)$, in orange, green and blue respectively with burst-sizes, $b = (2, 5, 10)$, and efficiency of abortion, $\omega = 0$. For each critical invasion threshold we compute the probability of invasion, by averaging 50,000 stochastic realisations up to time $t_{max} = 1000$, for the following proportions of permissive cells, $Q^* = (0.05, 0.1, 0.15 \ldots 1)$, shown in dots. Importantly, our numerical derived function of $\bar{I}$ (shown by dots) is in close agreement with our analytical approximation, equation (2.9), shown with lines.

3 DISCUSSION

Genes that cause abortive infection can have the dual and inseparable phenotype of causing post-segregational killing. We show that addictive abortive infection determinants act to stabilise the radically altruistic behaviour of suicide upon bacteriophage infection by killing cells that fail to inherit the cooperative trait. Although we specifically examine addictive abortive infection, post-segregational killing or addiction is likely to extend the existence conditions of *any* linked cooperative allele. The maintenance of altruistic suicide upon infection, and the bacterial population as a whole, critically depends on the prevalence of the cooperative trait; if too many uncooperative permissive cells emerge, then bacteriophage infections will become systemic resulting in the collapse of the population. Using a simple population biology model we show that when addictive abortive infection traits are borne by mobile elements (in this case a conjugative plasmid), addiction acts to suppresses the emergence of permissive cells thereby maintaining the cooperative locus at a higher prevalence than in its absence. The effect of addiction on the prevalence of the cooperative trait is most pronounced when rates of segregational loss are high, like those found in mixed plasmid populations, or when horizontal transfer is restricted, such low cell density environments. In our model, efficacious addiction, $\varepsilon < 10^{-2}$, may reduce the proportion of permissive cells at equilibrium by an order of magnitude. In these situations, if altruistic suicide were not linked to an addiction locus, bacterial populations would collapse. In an unstructured environment the proportion of permissive cells at equilibrium, Q^*, must not exceed the critical threshold $(1 - wb)/(b - wb)$, where b is the mean burst-size of a successful infection and $0 < w < 1$ is the efficacy of abortive infection. This threshold has an equivalent reproductive ratio, $R_0 = Q^* b + (1 - Q^*)wb$, which can be intuitively understood as the mean number of progeny phage produced by both permissive and abortive infections per infection. The deterministic, or mean-field, threshold for invasion limits the presence of permissive cells to a small fraction of the population given burst-sizes for lytic bacteriophage typically range between $100 < b < 300$. However, when permissive cells are rare or a minority at equilibrium, invasion of an initially rare phage depends heavily on the chance infection of a permissive cell. Using an individual-level model we numerically calculated the probability of invasion as a function of the proportion of permissive

cells. As predicted, at the mean-field threshold, the probability that phage invade is highly unlikely, with the probability of invasion being in the order of <1% when assuming absolute abortion (i.e. infection results in no phage progeny). Thus, stochastic effects relating to the rarity of invading phage mean that bacterial populations may tolerate significant subpopulations of permissive cells without undermining cooperative altruistic suicide.

(a) Replicon dynamics and addictive abortive infection

The population dynamics of bacterial cells are not always aligned with that of the replicons they bear. In the case of addictive abortive infection, within-host dynamics arising from reproductive competition between cognate plasmid replicons will likely influence both population and evolutionary dynamics. Within-host dynamics may arise whenever genotypically distinct replicons attempt to transmit within a single cellular lineage. A cell may come to bear multiple distinct plasmid replicons by either co-infection (i.e. super-infection) in mixed-plasmid populations or simply whenever a plasmid acquires a mutation that gives it a new biological identity. For the sake of mathematical tractability, our model focused simply on segregation and horizontal transfer of a single plasmid genotype and neglected complexities arising from plasmid reproductive biology. In the following sections we elaborate on these complexities and speculate on how addictive abortive infection may behave in the presence of competing cognate plasmids.

(i) Post-segregational killing may restrict plasmid- and chromosomal-defection

Plasmids have been theorised to promote bacterial cooperation by dispersing cooperative traits among otherwise unrelated individuals thereby ensuring relatedness at the level of the plasmid and its cooperative genes (Smith, 2001). In this scenario, like in our model, defectors emerge or are coerced into cooperation through loss or gain of a plasmid that bears a cooperative locus. An important limitation of this model is that defection is constrained to the cell-level, meaning defector female cells lacking the plasmid can be simply re-infected to rescue cooperation. This assumption is valid for defectors that emerge via segregational loss of the plasmid but naïve when considering defection at the plasmid-level (Mc Ginty *et al.*, 2011). If plasmid-defectors emerge through loss of the cooperative trait by mutation, recombination or deletion, replication control mechanisms acting on cognate origins lead to the rapid

segregation of each plasmid genotype into distinct lineages (for an in-depth discussion on this process see Chapter 3). This replication incompatibility and the supposed fitness advantage of defection mean that cooperation can no longer be rescued by re-infection.

Empirical and theoretical studies of plasmid co-infection have shown that plasmid-borne post-segregational killing determinants eliminate cognate replicons lacking immunity via host-killing upon segregation (Cooper & Heinemann, 2000; Rankin *et al.*, 2012; Chapter 3). When closely linked to a cooperative trait, post-segregational killing would act to stabilize cooperation by killing plasmid- or chromosomal-defectors that have lost the cooperative region. In the case of toxin-antitoxin based Abi determinants, defectors may only arise when expression of the toxin is lost but expression of the antitoxin maintained. Such a targeted mutation, which would most likely be a result of a point mutation or transposon insertion, may not confer an appreciable fitness advantage to the defector, meaning "defections" may be removed by genetic drift and not necessarily destabilise cooperation.

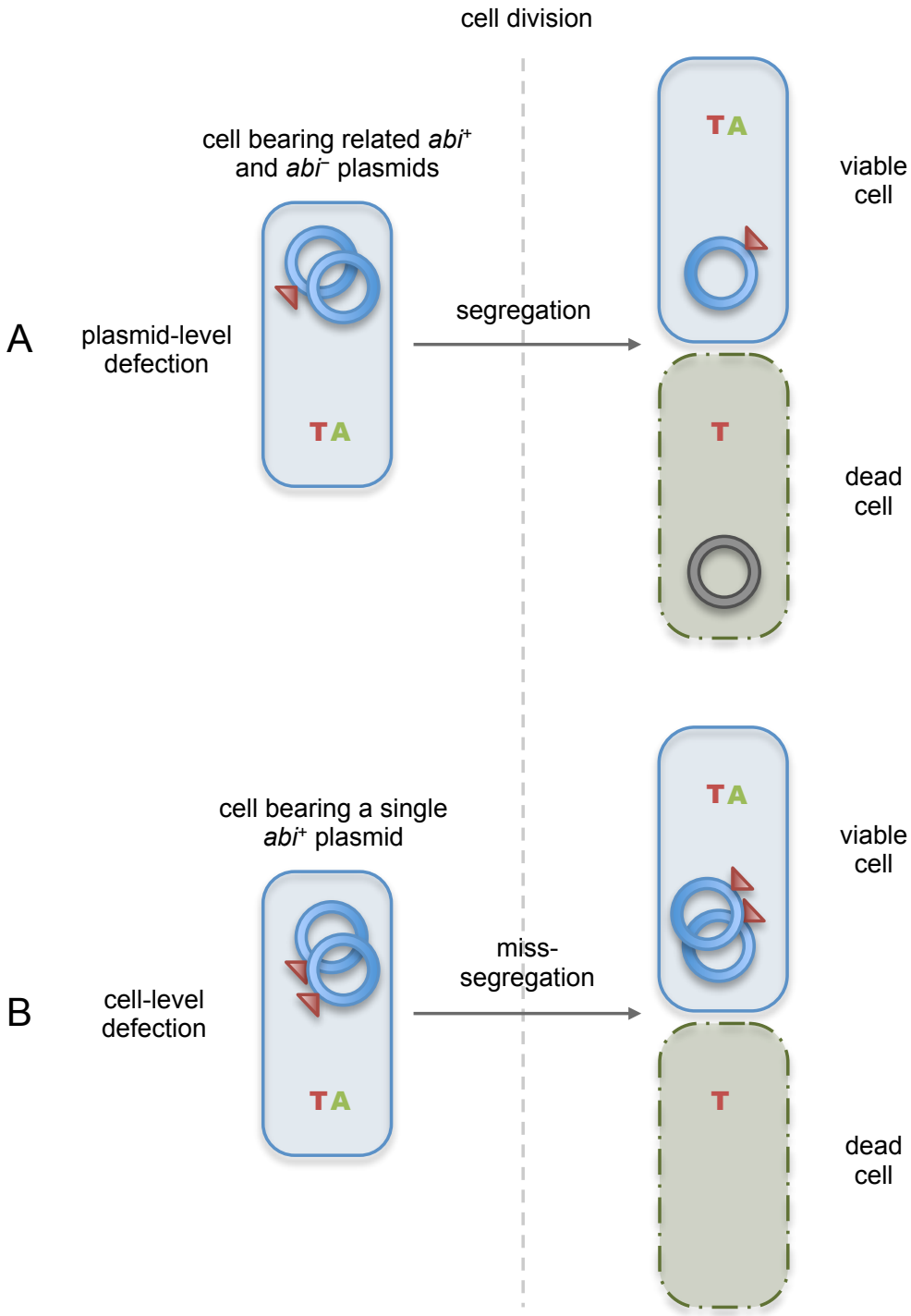

Figure 22. Plasmid-level and cell-level defection. (A) Plasmid-level defection occurs when a single plasmid copy loses the *abi* by mutation or recombination, then upon cell division plasmid copies may segregate to different daughters creating *abi*$^+$ and *abi*$^-$ lineages (B) Cell-level defection occurs when the *abi* locus fails to segregate to both daughters of cell division (miss-segregation). In both cases post-segregational killing removes *abi*$^-$ cheats.

(ii) Maintaining abortive infection in mixed plasmid populations

Plasmids that share cognate origins of replication segregate rapidly into distinct lineages. This reproductive *incompatibility* is due to plasmid copy control mechanisms and appears to be a general feature of plasmid biology (see Chapter 3). Incompatibility causes both near-isogenic mutants and distantly related replicons that possess cognate origins to rapidly segregate. In mixed-plasmid populations, incompatibility poses an existential threat to plasmid-mediated cooperation. Co-infected cells would rapidly generate daughter cells lacking the cooperative plasmid, meaning that the cooperative locus could not be maintained at a high prevalence in populations that contain competing plasmids. Plasmids bearing a post-segregational killing determinant have been shown to invade and *eliminate* plasmids lacking the determinant (Cooper & Heinemann, 2000; Chapter 3). In this scenario, plasmids lacking the post-segregational killing determinant are killed upon cell division when each plasmid eventually segregates into distinct daughter cells. In turn, this intragenomic conflict selects for cognate post-segregational killing determinants on competing plasmids, whom by acquiring it are immune to killing (Cooper *et al.*, 2010). Thus, co-infection and post-segregational killing might allow addictive abortive infection determinants to colonise, and be maintained in a diverse range of genetic backgrounds. In backgrounds where the plasmid is not faithfully inherited, selection would drive the cooperative locus onto replicons that are. In our model, horizontal transfer alone is sufficient for maintaining the cooperative plasmid at high prevalence (i.e. addiction is redundant), however, in the presence of competing plasmids, addiction would be crucial for ensuring segregants generated by reproductive conflicts in co-infected cells are killed. Thus, as plasmid-level reproductive conflicts emerge due to horizontal transfer into cells bearing a cognate plasmid (co-infection), addiction would be become of increasing importance as the rate of horizontal transfer increases.

(b) Evolution of addictive abortive infection and strong reciprocity

Strong reciprocity, where an individual helps others but punishes those that are not helping, is thought to be an important force leading to the evolution of cooperative and altruistic traits (Gintis, 2000; Boyd *et al.*, 2010). In cases of strong reciprocity, cooperation and punishment are costly acts that confer no fecundity advantage to the

focal individual but instead benefit the population as a whole. A fundamental problem concerning the evolution of strong reciprocity is that non-punishing cooperators are predicted to invade populations of punishing cooperators whereby non-punishing cooperators benefit from, but do not contribute to, the costly punishment of non-cooperators (i.e. the so called "tragedy of the commons" simply occurs at a different level). Theory predicts that strong reciprocity can evolve in structured environments if the cooperative and punishment traits are perfectly linked (i.e. the traits rarely segregate by recombination), meaning punishment is directed towards non-punishers (Lehmann *et al.*, 2007). Consistent with theory, experiments using *Pseudomonas aeruginosa*, which are suggested to cooperate by secreting iron-scavenging siderophores and punish by secreting bacteriocins, found that cooperative punishers may only invade when the two traits are tightly linked (Inglis *et al.*, 2014). As such, it is thought that the evolution of costly punishment is favoured only when it results in a direct or indirect fitness benefit to the focal individual (Lehmann *et al.*, 2007; Inglis *et al.*, 2014). Perhaps the most encouraging sign that addictive abortive infection may have evolved because of strong reciprocity is that both helping and punishment phenotypes are inseparable. The same toxin-antitoxin complex mediates both abortive infection and post-segregational killing. Because of this perfect linkage, non-cooperators are also non-punishers, meaning punishment is directed towards non-cooperators and non-punishers. To our knowledge, addictive abortive infection is the first known bacterial example of a perfectly linked punishment-cooperation locus.

The phenotypic properties of addictive abortive infection bear many similarities to other examples of strong reciprocity (both theoretical and empirical). Individuals both help others, by constitutively expressing proteins that promote cell death upon infection, and punish non-helpers that have lost the toxin-antitoxin operon through post-segregational killing. Although the overall phenotypic effects are similar, there are two important differences: 1. abortive infection exports an incorporeal public good, where abortion upon infection by a phage generates a phage-less environment. This is fundamentally different to other types of exported bacterial public goods which are typically tangible secreted goods, e.g. siderophores; 2. punishment is restricted to offspring of cells that defect, where uncooperative daughter cells are

implicitly recognised by their inability to neutralise the effect of the intracellular toxin, rather than any cell that is not bearing the abortive infection gene complex.

However, conversely, the combination of plasmid-mediated horizontal transfer and post segregational killing could be viewed in an analogous way to a secreted toxin with the advantage that cells are given an opportunity, albeit an ultimatum, to cooperate by maintaining the addictive abortive infection determinant. In this respect post-segregational killing may be a more nuanced approach to punishment than the secretion of bacteriocins. Instead of a scorched-earth approach, where bacteriocins render the environment uninhabitable to non-cooperators, cells are coerced into cooperating, albeit by pain of death. This difference could mean that addictive abortive infection is more readily acquired by conjugation and may rapidly spread through diverse genetic backgrounds.

Because of these differences, i.e. the incorporeal nature of the public good and confounding dynamics caused by plasmid population biology (e.g. conjugation and replication incompatibility), it is hard to extrapolate from current models of bacterial strong reciprocity to addictive abortive infection. Moreover, post-segregational killing is known to increase plasmid fitness during mixed infections (Cooper & Heinemann, 2005), meaning addiction may invade simply by conferring a direct fitness advantage to the plasmid. Thus, we cannot conclude that strong reciprocity is the evolutionary *raison d'etre* for addictive abortive infection determinants; however, our population biology model shows that linkage of post-segregation killing extends the existence conditions of abortive infection.

(c) Concluding remarks

Mobile genetic elements, like conjugative plasmids, permit certain alleles to transcend lineage boundaries that otherwise constrain chromosomal genes. In the case of abortive infection, horizontal transfer may potentially serve to increase assortment across members of the host-range of a bacteriophage. However, horizontal transfer alone may not be enough to ensure abortive infection is maintained at a sufficiently high prevalence to maintain the cooperative behaviour. Reproductive conflicts between related mobile genetic elements, such as copy control in vegetative plasmids,

means that stable vertical transmission in a new lineage is far from guaranteed. Over time, toxin-antitoxin mediated post-segregational killing resolves this within-host conflict by purging uncooperative elements that hinder maintenance of the trait at high prevalence and at the same time selecting for introgression of addictive abortive infection upon competing elements granting them immunity to punishment.

The core prediction of this chapter, that linked post-segregational killing determinants extend the existence conditions of abortive infection, is eminently testable. Although, addiction and abortive infection are genetically inseparable (i.e. dual phenotypes of a single genetic locus), the two phenotypes should be distinguishable by complementing antitoxin activity in *trans*. If for example, an addictive abortive infection determinant were borne by a conjugative plasmid, a chromosomally borne antitoxin would destroy post-segregational killing activity but not alter abortive infection (assuming gene dosage affects do not disrupt abortive infection). Observing the relative persistence of abortive infection in these two genetic backgrounds with respect to predation by bacteriophage would then determine the effect of linked post-segregational killing.

Hitherto, only a handful of abortive infection determinants have been observed to also kill miss-segregants, however, the phenomenon does not appear to be limited to a specific biochemical type of toxin-antitoxin system. Addictive abortive infection has been observed in: antisense systems, exclusion of T4 by *hok*/*sok* (Pecota & Wood, 1996); protein-protein systems, exclusion of P1 by *mazEF* (Hazan *et al.*, 2001; Hazan & Engelberg-Kulka, 2004); and, protein-RNA systems, broad-range exclusion by *toxIN* (Fineran *et al.*, 2009). This suggests the relationship between post-segregational killing and abortive infection has evolved multiple times and is thus likely to be a broadly occurring phenomenon.

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5 APPENDIX

(a) Equilibrium E_3

$$P_3^* = \frac{(c+s)(\omega b-1) - s\epsilon(b-1)}{b\beta(\omega-1)} \tag{5.1a}$$

$$A_3^* = -\frac{P_3^*(b-1)}{\omega b-1} \tag{5.1b}$$

$$V_3^* = \frac{k(1-c-s)(\omega b-1) + P_3^*\big(k\beta(\omega b-1) - b(\omega-1)\big)}{k\phi(\omega b-1)} \tag{5.1c}$$

(b) Phage invasion condition

Phage may invade at $V_3^* > 0$. Expanding equation (5.1) gives

$$V_3^* = \frac{kb\beta(1-c-s)(\omega b-1)(\omega-1) + \big((c+s)(\omega b-1) - s\epsilon(b-1)\big)\big(k\beta(\omega b-1) - b(\omega-1)\big)}{k\phi b\beta(\omega b-1)(\omega-1)} \tag{5.2}$$

Since the efficiency of abortion is constrained by $0 \leq \omega \leq 1$, and when assuming abortion limits systemic phage reproduction, $\omega b < 1$, the denominator of equation (5.2) is strictly positive. Therefore, the non-trivial condition for phage invasion is

$$kb\beta(1-c-s)(\omega b-1)(\omega-1) + \big((c+s)(\omega b-1) - s\epsilon(b-1)\big)\big(k\beta(\omega b-1) - b(\omega-1)\big) > 0. \tag{5.3}$$

Inequality (5.3) was confirmed using numerical methods (not shown).

(c) Derivation of $Q_3{}^*$

$$Q_3{}^* = \frac{P_3{}^*}{P_3{}^* + A_3{}^*} \tag{5.4a}$$

Given $A_3{}^* = -\frac{P_3{}^*(b-1)}{\omega b-1}$

$$Q_3{}^* = \frac{P_3{}^*}{P_3{}^* - \frac{P_3{}^*(b-1)}{\omega b-1}} \tag{5.4b}$$

$$Q_3{}^* = \frac{P_3{}^*}{\frac{P_3{}^*(\omega b-1)}{\omega b-1} - \frac{P_3{}^*(b-1)}{\omega b-1}} \tag{5.4c}$$

$$Q_3{}^* = \frac{P_3{}^*(\omega b-1)}{P_3{}^*(\omega b-1-(b-1))} \tag{5.4d}$$

$$Q_3{}^* = \frac{\omega b-1}{\omega b-b} \equiv \frac{1-\omega b}{b-\omega b} \tag{5.4e}$$

Alternatively, $Q_3{}^*$can be derived by evaluating the invasion criterion $\frac{dV}{dt}\frac{1}{V} > 0$ at E_3 assuming ϕ, $P_3{}^*$ and $A_3{}^*$ are non-negative.

$$\frac{dV}{dt}\frac{1}{V} = \phi(P_3{}^*(b-1) + A_3{}^*(\omega b-1)) > 0 \tag{5.5a}$$

$$P_3{}^*(b-1) + A_3{}^*(\omega b-1) > 0 \tag{5.5b}$$

$$\frac{P_3{}^*(b-1) + A_3{}^*(\omega b-1)}{P_3{}^* + A_3{}^*} > 0 \tag{5.5c}$$

$$Q_3{}^*(b-1) + (1-Q_3{}^*)(\omega b-1) > 0 \tag{5.5d}$$

$$Q_3{}^*(b-1) - Q_3{}^*(\omega b-1) > 1-\omega b \tag{5.5e}$$

$$Q_3{}^*(b-\omega b) > 1-\omega b \tag{5.5f}$$

$$Q_3{}^* > \frac{1-\omega b}{b-\omega b} \tag{5.5g}$$

(d) Derivation of inequality (2.5)

Starting with inequality (5.5)d

$$Q_3{}^*(b-1) + (1-Q_3{}^*)(\omega b - 1) > 0 \tag{5.6a}$$

$$Q_3{}^* b - Q_3{}^* + (1-Q_3{}^*)\omega b - 1 + Q_3{}^* > 0 \tag{5.6b}$$

$$Q_3{}^* b + (1-Q_3{}^*)\omega b > 1 \tag{5.6c}$$

(e) Numerical validation of individual-level model (2.6)

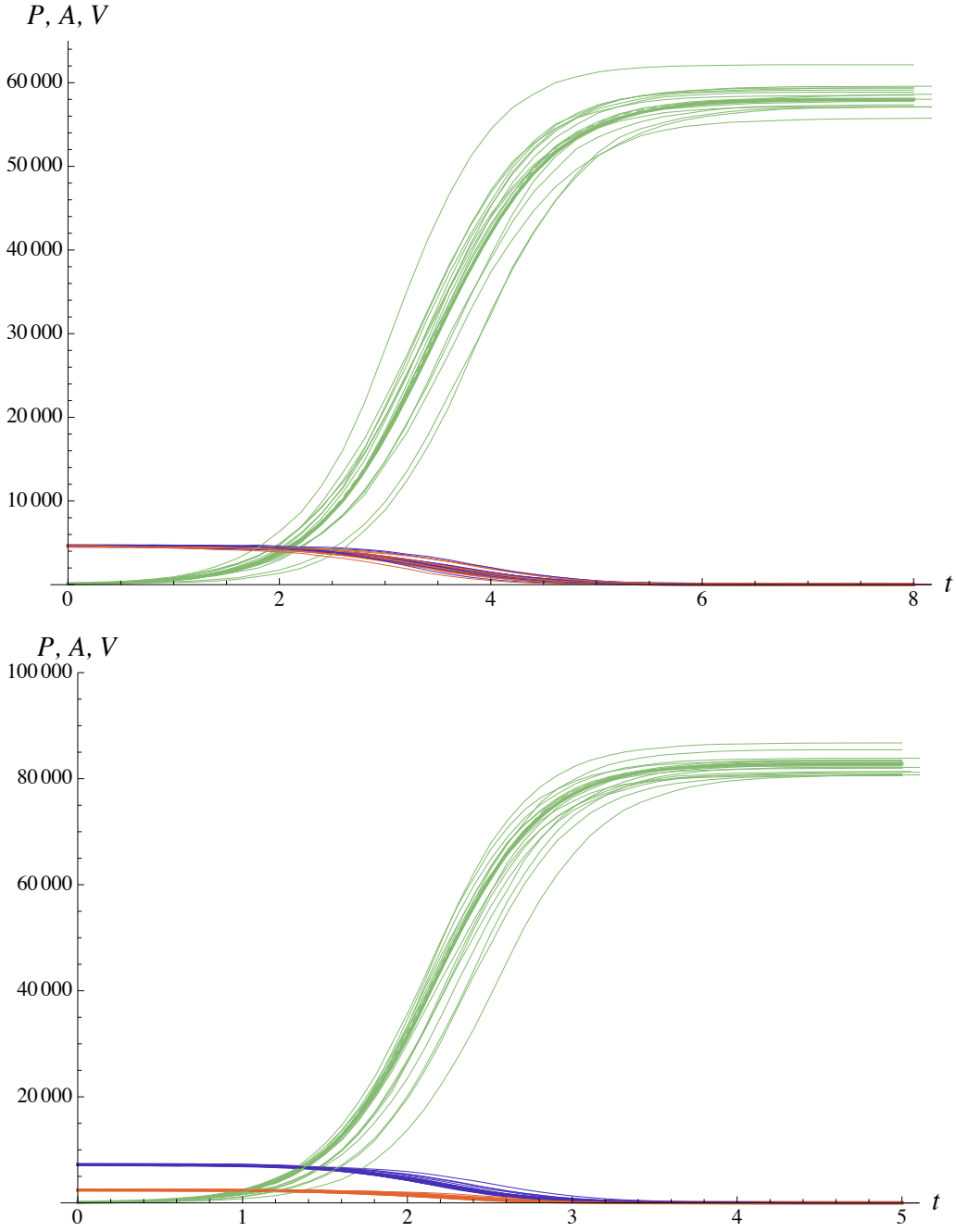

Figure A1. Stochastic integration of the individual-level model, (2.6), using Gillespie's direct method showing the average behaviour of model (2.6) is in close agreement with the mean-field population-level model, (2.2). Thick lines are population means for 5000 stochastic integrations of the individual-level model, dashed lines are population means predicted by the population-level model and thin lines are 16 random stochastic integrations. Permissive, abortive and virus are in blue, orange and green respectively. (top) $Q^* = 0.5$, $\beta = 1.62659\times10^{-5}$, $p(0) = 4626$, $a(0) = 4626$, $v(0) = 100$. **(bottom)** $Q^* = 0.75$, $\beta = 1.55997\times10^{-5}$, $p(0) = 7220$, $a(0) = 2407$, $v(0) = 100$.

(f) Parameters used for numerically determining the probability of invasion

Q^*	β
0.05	1.8590×10^{-5}
0.1	1.7852×10^{-5}
0.15	1.7508×10^{-5}
0.2	1.7265×10^{-5}
0.25	1.7063×10^{-5}
0.3	1.6884×10^{-5}
0.35	1.6717×10^{-5}
0.4	1.6561×10^{-5}
0.45	1.6411×10^{-5}
0.5	1.6266×10^{-5}
0.55	1.6126×10^{-5}
0.6	1.5990×10^{-5}
0.65	1.5857×10^{-5}
0.7	1.5727×10^{-5}
0.75	1.5600×10^{-5}
0.8	1.5475×10^{-5}
0.85	1.5353×10^{-5}
0.9	1.5233×10^{-5}
0.95	1.5116×10^{-5}
1	1.5×10^{-5}

Table A2. Rates of horizontal transfer, β, required for varying proportions of permissive cells, Q^*, with $k = 10^4$, $c = 0.1$, $s = 0.05$, $\varepsilon = 0.01$ and $\phi = 5\times10^{-5}$.

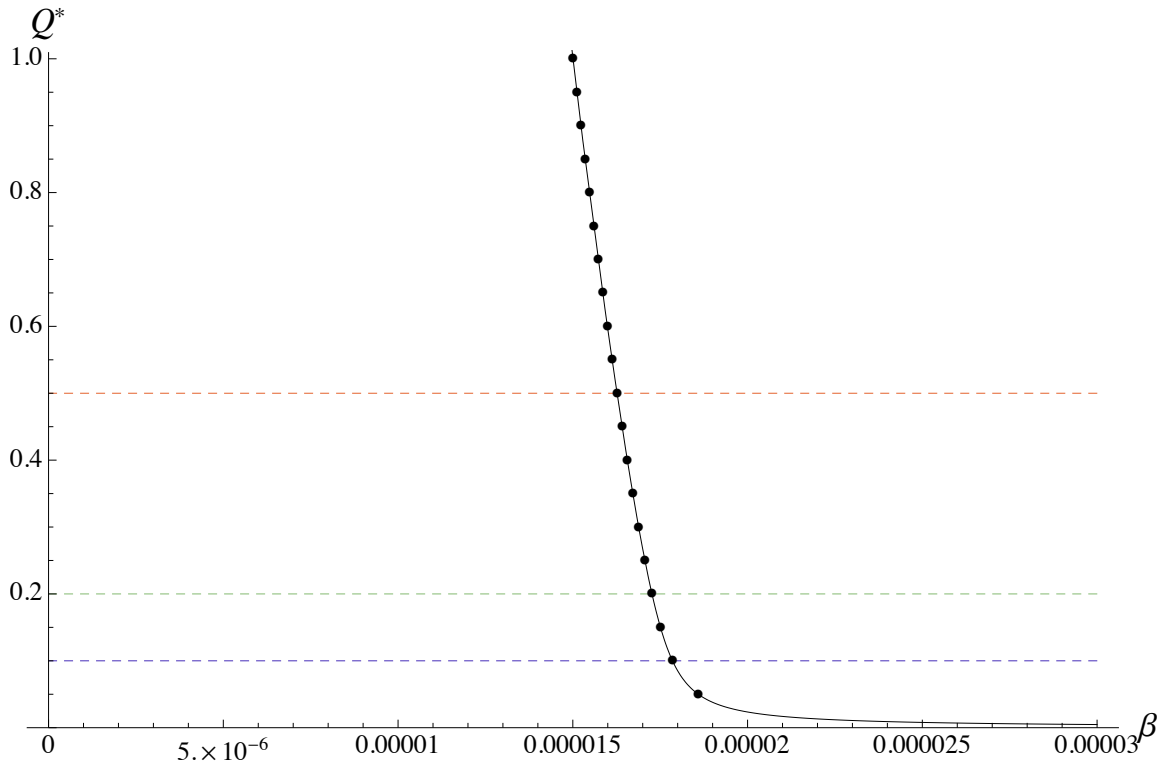

Figure A2. Simulation parameter-space with Q^* as a function of horizontal transfer, β. Black dots are solutions to Q^* = (0.05, 0.1, 0.15 … 1) (values for β and remaining parameters are the same as in Table A2). The critical thresholds $\tilde{Q} = (0.5, 0.2, 0.1)$ are in orange, green and blue respectively with burst-sizes $b = (2, 5, 10)$, and efficiency of abortion, $\omega = 0$.

CHAPTER 5

Discussion

1 SUMMARY OF RESEARCH

The ubiquitous presence of addiction complexes on bacterial replicons poses an intriguing evolutionary conundrum, namely, how can a trait whose principal phenotype, host-killing, be so pervasive? This thesis explored how addiction complexes affect the population dynamics of chromosomes and plasmids that carry them. We developed and analysed mathematical models of three interesting cases of addiction complex population biology: 1. the emergence of coupled toxin antitoxin gene complexes and intragenomic conflict, 2. within-host competition between competing plasmids, 3. punishment of segregating non-cooperative alleles. Collectively, these three cases examine emergence at the level of the operon, replicon (chromosome and plasmid) and community (i.e. sociobiological traits).

The theoretical models developed herein were novel in their approach owing to their focus on the ecology of replicons (i.e. population dynamics of plasmids, chromosomes and phage interacting in ecosystems as small as a single cell to as large as populations of cells). This replicon-centric approach allowed us to examine adaptation at the level of the replicon, which in turn generated novel hypotheses as to how addiction complexes may drive bacterial genome dynamics. Importantly, each model developed herein qualitatively predicted many observed behaviours that existing models do not. The following is a summary of the key findings of this thesis.

(a) Emergence of addiction complexes and intragenomic conflict

In Chapter 2, we first extended earlier results demonstrating that addiction complexes can spread on plasmids in spatially structured environments as a result of kin-selection (Mongold, 1992; Mochizuki *et al.*, 2006). We then considered the emergence of addiction complexes on plasmids from previously unlinked toxin and antitoxin genes. We found that one of these traits must offer at least initially a direct advantage in some but not all environments encountered by the evolving plasmid population. Furthermore, we found during the spread of an addictive plasmid, the establishment of an addiction complex on the host chromosome is favoured by protecting the cell from plasmid-mediated host killing. If this addiction complex is costly to the host cell, selection on this gene pair is likely to be reversed as soon as the addictive plasmid is sent sufficiently into decline, in turn favouring the subsequent

invasion of a non-addictive plasmid and thus opening the potential for 'rock-paper-scissors' cycling coexistence. This result suggests that intragenomic conflict could be sufficient to select deleterious genes on chromosomes and helps to explain the previously perplexing observation that many addiction complexes are found on bacterial chromosomes (Tsilibaris *et al.*, 2007).

(b) Plasmid co-infection and addiction mediated within-host competition

In Chapter 3, we explicitly account for plasmid co-infection and demonstrate that kin-selection is unnecessary for explaining the evolution and population dynamics of addiction complexes. We found that plasmid co-infection and horizontal gene transfer were sufficient for the emergence and coexistence of distinct mal-adaptive plasmid replicons. In such plasmid ecosystems, addictive plasmids outcompete plasmids lacking the addiction complex. Moreover, we find that addictive plasmids can even outcompete host-adaptive plasmids. We predict that within-host competition selects addiction complexes upon reproductively competing replicons and therefore can be viewed as an essential feature of bacterial replicons.

(c) Evolutionary relationship between addiction and abortive infection

In Chapter 4, we examined the relationship between the dual and inseparable phenotype of many addiction complexes – abortive infection. First, using a model of plasmid, cell and phage population dynamics, we showed that addictive abortive infection determinants act to stabilise the radically altruistic behaviour of suicide upon bacteriophage infection by killing cells that fail to inherit the cooperative trait. We conjecture that addiction may also act to prevent horizontally mobile cheaters from displacing horizontally mobile cooperators. Although we specifically examine abortive infection, addiction is likely to extend the existence conditions of *any* linked cooperative allele. Second, using an individual-level analogue of the population-level model we examined stochastic effects. We found that by suppressing cheats, linked addiction significantly reduced the probability of population collapse. To our knowledge, addictive abortive infection is the first example of a perfectly linked cooperation and punishment locus.

2 REVISITING CONCEPTS

The models developed in this thesis explored the relationship between addiction complexes and the population biology of the replicons that carried them. In all cases, this replicon-centric approach was needed to explain the population biology of addiction complexes. In the following sections, we step back from the details of addiction complexes and revisit notions of bacterial and plasmid evolution in light of the themes revealed by our models.

(a) Bacterial genome evolution

It is commonly held that bacterial genomes comprise an essential genetic core and an assortment of dispensable accessory genes (Medini *et al.*, 2005); where accessory genes are locally adaptive and are not essential in all environments. It has been proposed that bacteria sample genes from mobile genetic elements and retain traits that are locally adaptive. Our findings suggest that some assumed "accessory" genes borne by mobile elements might not be locally adaptive to an unknown environment but globally mal-adaptive with respect to the cell.

A common thread found in this thesis is how bacterial genomes evolve through the acquisition and loss of mobile replicons, and whose presence or absence radically alters the fitness of the cell's constituent replicons. In this sense, a bacterial genome can be thought of as a collection of cooperative and self-interested replicating entities. As demonstrated in Chapters 2, 3 and 4, the interactions between replicons (i.e. ecology) largely determined the evolutionary success of a trait of interest (in our case addiction complexes). For example, in Chapter 3 we show that even host-adaptive plasmids are unable to invade populations where cognate addiction complex carrying plasmids reside. This example is an important reminder that fitness must be considered at the level of the replicon (i.e. linkage group) and that host-adaptation is not sufficient to predict plasmid success.

The "bag of replicons" formulation means that "accessory" genes may actually be "core" genes at the replicon level of selection and their presence or absence in a particular lineage may not necessarily be predicted by the relationship to the cell (i.e. harmful or beneficial), but their relationship to other elements in the cytoplasm (i.e. replicon-level adaptions such as competition determinants). Importantly, this view

means chromosomal introgression is not an evolutionary "endgame" for all mobile genes, and thus, genes shared across bacterial lineages are not the only genes that are emblematic of HGT. Instead, many horizontally mobile genes will transmit through bacterial populations, and contribute to the phenotype of the host, but are never selected upon chromosomes. Moreover, genes that have introgressed into chromosomes may have been selected upon mobile elements for entirely different reasons, meaning their current utility when borne by a chromosome may not predict why they were initially borne by a mobile element. Comparative genomic methods perhaps reinforce the idea of horizontal gene transfer as the transfer *and* introgression of genes. This view unfortunately neglects a whole class of mobile genes whose adaptation occurs at the level of the replicon.

Formulating genomes as "bags of replicons" is perhaps more predictive of evolutionary outcomes than simply describing genes as "core" and "accessory". However, the use of this formulation is limited by the difficulty of observing how replicons assort themselves within populations of cells. Although, an increasing number of naturally occurring plasmids have been sequenced by meta-genomic studies, their ecological context is still not known.

In summary, our results suggest that in the short term, bacterial evolution is driven by the ecological dynamics of the population's constitutive replicons. An intuitive example of this is that antibiotic resistance is far more likely to be acquired via acquisition of a transmissible element than evolve *de novo* (J. Davies & D. Davies, 2010). The success of a plasmid-carried antibiotic resistance determinant is not simply predicted by its utility to the host. Instead, its utility must lie with the mobile replicon that bears it; if this replicon is unable to outcompete like-replicons then the resistance determinant may not be able to transmit to new lineages.

(b) Plasmid fitness

Despite the ubiquity of plasmids in nature, there is no consistent understanding of what makes a 'successful' plasmid. Current mathematical models of plasmid population biology (e.g. Stewart & Levin, 1977; Lili *et al.*, 2007) predict, given empirical estimates of conjugal transfer rates, plasmids could only exist by bearing

host beneficial alleles (i.e. genes that increased vegetative reproduction). This observation has led to what is referred to as the plasmid paradox – "mal-adaptive plasmids should be lost to purifying selection, whereas adaptive genes carried on plasmids should be integrated into the bacterial chromosome" (Harrison & Brockhurst, 2012). This framing of plasmid fitness, however, neglects interactions that take place either at the level of the group or within the host. Borrowing from the work presented in this thesis, this section considers more generally plasmid fitness in terms of replicon-replicon interactions.

(i) Combinatorial genetic backgrounds

Accounting for co-infection of mobile elements requires the consideration of complex within-host dynamics. In Chapter 3, we explored the simplest possible co-infection scenario, that of a mutant and near-isogenic wild-type plasmid. In this case, competitive within-host interactions (replication control) significantly reduce plasmid fecundity in the presence of the competitor. Moreover, the effect of addiction fell exclusively upon segregating competitor plasmids, meaning the incidence of mortality of the competitor increased. Neither effect occurs in singularly infected cells. Furthermore, competitive within-host dynamics between plasmids is not limited to vertical transfer. Plasmid conjugal transfer is known to vary dramatically in the presence of co-infecting mobile elements (Dionisio *et al.*, 2002), however, the details of this interference remain unstudied.

Thus, the fitness of a plasmid is dependent on the ecological interactions it is likely to encounter within the receiving population. In this respect, plasmid fitness becomes combinatorial. For example, consider a population of bacteria in which three distinct plasmid genotypes, A, B and C, co-exist. At any given time, a focal plasmid may either exclusively inhabit a cell or share it with 1 or 2 different plasmid genotypes. Within-host interactions mean the fitness of a focal plasmid differs depending on genetic background, yielding a combinatorial set of unique fitness functions (see Table 4 for an enumeration of fitness functions). Each fitness function is unique to the focal plasmid i.e. $f_B(A) \neq f_B(C)$, whilst the fitness of a focal plasmid will vary depending on which competing elements are present i.e. $f_A(C) \neq f_B(C)$. In a combinatorial model of plasmid fitness, mean plasmid fitness becomes the sum of

fitness across each genotypic background *pro rata* the proportion of time spent in each background (van Baalen & Sabelis, 1995).

Presence or absence of plasmid			Fitness functions of plasmid		
A	**B**	**C**	$\boldsymbol{f(A)}$	$\boldsymbol{f(B)}$	$\boldsymbol{f(C)}$
Yes	Yes	Yes	$f_{BC}(A)$	$f_{AC}(B)$	$f_{AB}(C)$
Yes	Yes	No	$f_B(A)$	$f_A(B)$	-
Yes	No	Yes	$f_C(A)$	-	$f_A(C)$
Yes	No	No	$f_\emptyset(A)$	-	-
No	Yes	Yes	-	$f_C(B)$	$f_B(C)$
No	Yes	No	-	$f_\emptyset(B)$	-
No	No	Yes	-	-	$f_\emptyset(C)$
No	No	No	-	-	-

Table 4. An enumeration of combinatorial fitness functions for 3 co-infecting plasmids. The first three columns describe the genetic background of a cell (i.e. whether it bears a plasmid genotype) whilst the remaining columns describe the fitness functions. The fitness function $\boldsymbol{f_B(A)}$ means the fitness of plasmid A in a cell co-infected with plasmid B, analogously, $\boldsymbol{f_{AB}(C)}$ means the fitness of plasmid C in a cell co-infected by plasmids A and B, whilst $\boldsymbol{f_\emptyset(B)}$ means the fitness of plasmid B in a host lacking other plasmid genotypes.

The practicality of combinatorial models is limited by the difficulty of modelling within-host fitness (Alizon, 2013). Epidemiological co-infection models, assume parasites transmit horizontally and have no vertical mode of reproduction (for a recent review of models see Alizon, 2013). In the case of vertically and horizontally transmitting parasites, like plasmids, trade-offs emerge between the cost of bearing a competition determinant and rate of vertical reproduction. This relationship means that the fitness of a plasmid cannot be decomposed into linearly separable vertical and horizontal fitness components as in the model of Lipsitch et al. (1995). This is by no means the only difficulty with co-infection models of vertically and horizontally transmitting parasites. Within-host frequency-dependent effects (i.e. the density of each parasite) may influence relative fitness. For example, plasmids *oriT*s may compete for relaxase and thus relative replicon concentration could be important.

(ii) Co-evolutionary interactions

In Chapter 2, we demonstrated co-evolutionary dynamics between chromosomal and plasmid replicons. This dynamic was driven by replicon-level conflicts (intragenomic conflicts) arising from the frequent segregation of addiction complex-carrying plasmids. Such conflicts are likely to be a common feature of bacterial replicon population biology; meaning a co-evolutionary approach to studying the evolution of bacterial replicons is required (Harrison & Brockhurst, 2012).

Plasmids are known to co-evolve with their hosts. For example, Dahlberg and Chao (2003) showed that cost of plasmid carriage can be ameliorated after 1100 generations without positive selection for plasmid carried traits. These studies reinforce the narrative that plasmids, in time, evolve to become benign commensals or beneficial secondary chromosomes (mutualists). Conversely, evolution towards an a-virulent state suggests that the experimental system either biased against horizontal transmission of the plasmid, or the environmental conditions of culturing system amplified the cost of the plasmid. In summary, plasmid-host co-evolutionary experiments tend to suggest that the evolutionary endpoint for plasmids is domestication, however, the existence of a multitude of cryptic and selfish parasitic genetic elements suggests that empirical studies are biased because of the lack of ecosystem complexity available in near monoculture conditions of the laboratory.

Few studies have examined the co-evolution of plasmids with other cellular replicons (e.g. plasmids and phage). Conflicts between replicons arise due to competing modes of reproduction. In the case of plasmids, one conflict arises due to reproductive self-restraint; however, other conflicts may emerge through indirect interactions that are transient in nature. For example, the carriage of some plasmids permit infection by lytic bacteriophage (male-specific bacteriophage infection) whereby phage attach at and enter via the conjugal pilli (Birge, 2006). This interaction should generate antagonistic co-evolutionary dynamics between the plasmid, phage and cell. The cell may respond with counter-measures directed at either the plasmid or phage, and in turn, the plasmid must contend with host counter-measures and a trade-off between horizontal gene transfer and phage predation.

Within-host conflicts are often intense but transient in nature. For example, co-infecting cognate plasmids quickly segregate to distinct lineages meaning the co-infected state is not stably maintained. This transiency in conflict means that it is difficult to unravel the ecological dynamics between bacterial replicons *in vitro*. Furthermore, metagenomic sequencing techniques are unable to resolve which replicons co-inhabit a single cytoplasm, and thus, our understanding of replicon ecology is unlikely to advance significantly from only sequencing ecosystems.

Perhaps the most promising pathway towards understanding the ecology within bacterial cells lies in observing conflicts indirectly by identifying genetic signatures of past or present conflict. It has been proposed that CRISPR target sequences provide a interaction map between antagonistically co-evolving replicons and can be used to infer the ecological relationship between replicons (Vale & Little, 2010). This idea can be extended to addiction complexes. If two replicons co-inhabit a single population and only one has an addiction complex, this would imply that replicons are not in reproductive conflict. That is, both replicons never co-inhabit a single cytoplasm, or if they do, are not in reproductive conflict. Conversely, if both replicons carried an addiction complex, it is not sufficient to prove a conflict between these elements exists as the addiction complex may be deployed against a third unknown replicon.

(iii) Empirical estimates of vertical and horizontal transfer

It is almost certain that co-evolutionary interactions for which conjugative plasmids are highly adapted to *in situ* are for the most part absent in *in vitro* observations of plasmid evolution and population dynamics. *In vitro* studies examine a narrow combination of environmental, physiological and genetic backgrounds making it hard to draw general conclusions as to the existence conditions of plasmids. For example, conjugation exhibits a high degree of phenotypic plasticity with respect to: local cell density (Turner, 2004); the reproductive state of the host (Haft *et al.*, 2009); and, the presence of competing or amplifying genetic elements (Dionisio *et al.*, 2002). If we assume *in vitro* derived estimates of plasmid conjugation rates are representative of *in situ* rates, conjugative plasmids would have to confer a fitness advantage to the host, while conjugation itself would be susceptible to purifying selection unless the act of

transfer also conferred a benefit to the focal cell such as increasing assortment of cooperative alleles and thus facilitating cooperative behaviours (Dimitriu *et al.*, 2014). In this scenario, autonomous forms of genetic reproduction, such as conjugation, are implied to have evolved to increase vertical reproduction of other replicons and in the case of plasmids their own vertical reproduction.

Interestingly, of the plasmids that have been identified thus far, vertical transfer appears to be crucial to their persistence. Indeed, to our knowledge, all known natural plasmid-like replicons are capable of faithful vertical inheritance in at least some species of bacteria. However, *in vitro* isolation entails successive population bottlenecks (clonal isolation by successive dilution) that would bias against replicons that are unable to reproduce vertically with the cell. If plasmid-like replicons that reproduce solely by horizontal transfer were to exist, it is unlikely that standard culturing techniques would capture them. However, here is where metagenomic approaches might be useful for ecosystem level studies.

3 FUTURE WORK

Considering bacterial genomes as the ecology of its constituent replicons is promising framework for understanding bacterial evolution. There are many techniques developed in related fields, predominately in epidemiology and ecological food webs, which are yet to be applied to the evolution of bacterial genomes. Although many avenues of research exist, the following research topics are perhaps some of the most interesting.

(a) Within-host reproduction models

Current models of co-infection do not capture the relative concentration of plasmid backbones present in each cell but rather the presence or absence of a plasmid genotype within a cell. Plasmid segregation is in part frequency-dependent e.g. if the relative plasmid concentration is 14:1 then segregation is guaranteed. Modelling frequency dependency is an important step towards complete models of plasmid population biology and will likely lead to a better understanding of reproductive trade-offs between plasmids and their hosts. Work in this direction has already commenced in related fields (Sofonea *et al.*, 2015).

(b) Co-evolutionary models of plasmid-host and plasmid-plasmid dynamics

Current mathematical models of plasmid population dynamics assume their traits to be static, however, they most likely change in response to ecological dynamics driven in part by competing replicons. Eco-evolutionary frameworks, such as adaptive dynamics and the epidemiology price equation (Dieckmann, 2002; Alizon, 2009) could be used to explore how a number of plasmid phenotypes (e.g. incompatibility) evolve in response to plasmid-plasmid and plasmid-host interactions. This line of investigation might help explain why some plasmids are highly infectious and virulent while others are immobile and benign.

(c) Plasmid population dynamics in non-naïve genetic backgrounds

As discussed in section 2, plasmid fitness varies depending on the genetic background of its host. Although interactions between a mutant plasmid and its isogenic wild-type sibling are extremely important, interactions between other replicons present in the cell could also influence invasion outcomes. Extending models to include multiple replicon types would build towards a "systems" level model of replicon population biology. At the systems level, more complex behaviours could be distinguished such as mutualistic relationships e.g. co-transmission of replicons bearing complementary genes.

4 CONCLUDING REMARKS

This thesis used a replicon-level approach for examining how phenotypes of toxin-antitoxin systems influence the evolution of bacterial genomes. By focusing on replicon population dynamics we were able to delineate between selection acting at the level of the cell or at the level of elements like plasmids. This approach generated a number of alternative hypotheses as to how addiction complexes persist in environmental populations which are not predicted using current state-of-the models (Mochizuki *et al.*, 2006; Cooper *et al.*, 2010). Understanding bacterial replicons at a systems-level is likely to greatly increase our understanding of how bacterial genomes evolve and the dynamics that decide upon which replicon a gene is adaptive.

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